# $Ca^{2+}$ AND $Na^{+}$ TRANSPORT BY CHROMAFFIN GRANULES OF THE ADRENAL MEDULLA

by

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Thesis for the Degree of
Doctor of Philosophy


University of Edinburgh
1989

*For my Family*

## *Declaration*

**The experimental work presented in this thesis was completed between October 1985 and September 1988. I declare that it is my own, and that this thesis has been composed by myself.**

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***Abstract***

Chromaffin granules, the secretory organelles of the adrenal medulla, store and secrete catecholamines, proteins and nucleotides. Their very high internal catecholamine concentrations are achieved by a chemiosmotic coupling between an inwardly directed, electrogenic $H^+$-translocating adenosine triphosphatase ($H^+$-ATPase) and a separate catecholamine/$H^+$ exchanger. $Ca^{2+}$ is also a major component of the granule matrix, its nominal concentration being 20-30mM. The mechanism of $Ca^{2+}$ uptake has hitherto only been partially characterised. $Ca^{2+}$ uptake via electroneutral $Ca^{2+}/Na^+$ exchange has been demonstrated in resealed membrane "ghosts" and intact granules (Phillips (1981), *Biochem. J.* **200**: 99-107; Krieger-Brauer & Gratzl (1982), *Biochem. Biophys. Acta.* **691**: 61-70). To sustain $Ca^{2+}$ uptake it is therefore necessary for granules to have an independent mechanism of $Na^+$ uptake.

In this thesis I show that chromaffin granules possess a novel amiloride-sensitive $Na^+/H^+$ antiporter in their membranes. This explains why $Na^+$ is mildly inhibitory to catecholamine transport (which is driven principally by the transmembrane pH difference generated by the ATPase). $Na^+/H^+$ antiport activity has been assayed using a variety of techniques: direct assay of $^{22}Na^+$ accumulation in response to a transmembrane pH difference ($\Delta$pH) generated by a rapid increase in external pH, or by ATP hydrolysis; generation of a $\Delta$pH (measured with a fluorescent probe) in response to an imposed $Na^+$ gradient; loss of a pH gradient due to $Na^+$ uptake; and $Na^+/Na^+$ exchange. The antiporter has a relatively high Michaelis constant ($K_m$) for extragranular $Na^+$ (4.7mM at pH 7.0 determined from fluorescence experiments) and is inhibited competitively by the diuretic drug amiloride, a well known inhibitor of plasma membrane $Na^+/H^+$ antiporters, with an apparent inhibition constant ($K_i$) of 0.26mM.

I have also measured the total and free concentrations of the inorganic cations $Na^+$ and $K^+$ inside the granule matrix. The total concentrations were found to vary depending on the ionic composition of the isolation buffer used to prepare the granules, with the lowest values being obtained with nominally $Na^+$ and $K^+$-free buffered sucrose solutions. The activity coefficients of both ions were found to be about 0.8, indicating that most of the $Na^+$ and $K^+$ is free within the matrix. The free concentration of $Ca^{2+}$, however, was found to be approximately 5μM, a value markedly lower than the previously measured total concentration of 20mM (Phillips *et al.*, (1977), *Neuroscience*, **2**: 147-152). In other words, its free concentration is only 0.03% of the total.

Because monovalent ion redistribution occurs during granule isolation, the concentrations of $Na^+$, $K^+$ and $Ca^{2+}$ measured *in vitro* cannot be extrapolated to the intact cell *in situ.*

Using $Na^+$-loaded resealed membrane "ghosts", and using $Ca^{2+}$ buffers to achieve various extravesicular free $Ca^{2+}$ concentrations, I have determined that the apparent $K_m$ for $Ca^{2+}$ uptake during $Ca^{2+}/Na^+$ exchange is about 1μM, with maximal rates of $Ca^{2+}$ uptake of the order of 1-2nmoles.$mg^{-1}$.$sec^{-1}$. In "ghosts", the activity of the $Na^+/H^+$ antiporter described above can be used to couple $Ca^{2+}$ uptake via $Ca^{2+}/Na^+$ exchange to electrogenic proton translocation via the granule membrane ATPase. Therefore, $Ca^{2+}$ uptake can be indirectly linked to the proton pump.

However, under conditions designed to mimic the environment of a granule in the cytosol of a chromaffin cell, only very low amounts of $Na^+$ could be accumulated within the granule matrix. Consequently, measured rates of $Ca^{2+}$ accumulation are also low. Under such circumstances, the granules seem unlikely to play a major role in calcium homeostasis in the intact cell.

## *Acknowledgements*

I am indebted to my supervisors, Drs. John H. Phillips and David K. Apps for their excellent guidance, supervision and scientific example over the past three and a half years; in particular I would like to thank them for their unfailing enthusiasm and support.

Grateful thanks also to Judith Percy, Kate Dry and the other members of the laboratory for their help and friendship; thank you also to my colleages and friends in the Department of Biochemistry, University of Edinburgh who helped to make my stay in Edinburgh a very enjoyable one.

I am also very pleased to acknowledge the assistance of Bülent Tugal and Anne Ward during the production of this thesis.

Finally, this work was supported by a Science and Engineering Research Council studentship to me, and this is gratefully acknowledged.

*Abbreviations Used*

| | |
|---|---|
| $[A^+]_i$, $[A^+]_o$, | free concentration of the ion $A^+$ either inside the granule or "ghost" matrix, or in the supporting medium |
| **ACh** | acetylcholine |
| **ACMA** | 9-amino-6-chloro-2-methoxyacridine |
| **ATP** | adenosine 5'-triphosphate |
| **ATPase** | adenosine 5'-triphosphatase |
| **c.p.m.** | counts per minute |
| $C_{12}E_8$ | octaethyleneglycoldodecyl ether |
| **d.p.m.** | disintegrations per minute |
| **DβH** | dopamine β-hydroxylase |
| **EDTA** | ethylenediamine tetraacetic acid |
| **EGTA** | 1,2-di(2-aminoethoxy)ethane-N,N,$N^1$,$N^1$-tetraacetic acid |
| **EIPA** | ethylisopropylamiloride |
| **FCCP** | carbonyl cyanide p-trifluoromethoxyphenylhydrazone |
| **HEDTA** | N-hydroxyethylethylene-diamine triacetic acid |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazineethanesulphonate |
| **MES** | 2[N-Morpholino]ethanesulphonate |
| **NEM** | N-ethylmaleimide |
| **NMR** | nuclear magnetic resonance |
| **NTA** | nitrilotriacetic acid |
| **Q** | percentage fluorescence quenching |
| **Q*** | percentage fluorescence quenching after ionophore addition |
| **r.p.m.** | revolutions per minute |
| **TMA** | tetramethylammonium hydroxide |
| **tris** | tris[hydroxymethyl]aminomethane |
| $v_i$ | internal volume of granules or "ghosts" |
| $v_o$ | volume of suspension medium |

| | |
|---|---|
| $\Delta pH$ | transmembrane proton concentration gradient |
| $\Delta\psi$ | transmembrane electrical potential gradient |
| $\Delta\tilde{\mu}_{H^+}$ | transmembrane electrochemical proton gradient |
| **5HT** | 5-hydroxytryptamine |

# *Contents*

*List of Tables*

## *Publications*

The following publications contain work presented in this thesis.

**(1)** Haigh, J. R. and Phillips, J. H. (1989) A sodium/proton antiporter in chromaffin granule membranes. *Biochem. J.* **257**: 499-507.

**(2)** Haigh, J. R., Parris, R. and Phillips, J. H. (1989) Free concentrations of sodium, potassium and calcium in chromaffin granules. *Biochem. J.* (in the press).

*Chapter One*

# *Introduction*

## *1.1 Biology of Chromaffin Cells*

### *1.1.1 Catecholamine-Storing Chromaffin Cells in the Adrenal Medulla*

The mammalian adrenal gland consists of two morphologically different tissues, the medulla and the cortex, which can be observed following a lateral section of the gland. The inner pink medulla is surrounded by a 3-5mm thick brown cortex, the latter being encased in a capsule of connective tissue. Embryologically, the cortex is of mesodermal and the medulla of ectodermal origin, the cells of both tissues developing from cells in the neural crest.

The adrenal medulla is a highly vascular tissue, having a dual blood supply to the two types of catecholamine-storing cells - adrenaline and noradrenaline containing cells. Most cells within the adrenal medulla synthesise and store adrenaline; a small number synthesise and store noradrenaline. However, the proportion of each type of cell varies from one species to another. These specialised cells were called **chromaffin cells** by Alfred Kohn at the beginning of the twentieth century and owe their name to the reaction of adrenaline in these cells to chromium salts, which produces a yellowish brown colour. A large number of small electron-dense vesicles which store adrenaline and noradrenaline are found in the chromaffin cell cytoplasm. These vesicles are called **chromaffin granules** and their presence and electron density allows one to discriminate between the two types of adrenal medullary cell: in electron micrographs the noradrenaline-containing granules appear more electron-dense with shrunken cores. This results from the complex between noradrenaline and the fixing agent glutaraldehyde (Coupland & Hopwood, 1966).

### *1.1.2 Biosynthesis of Catecholamines*

Within the chromaffin cell, a number of biosynthetic steps are required for

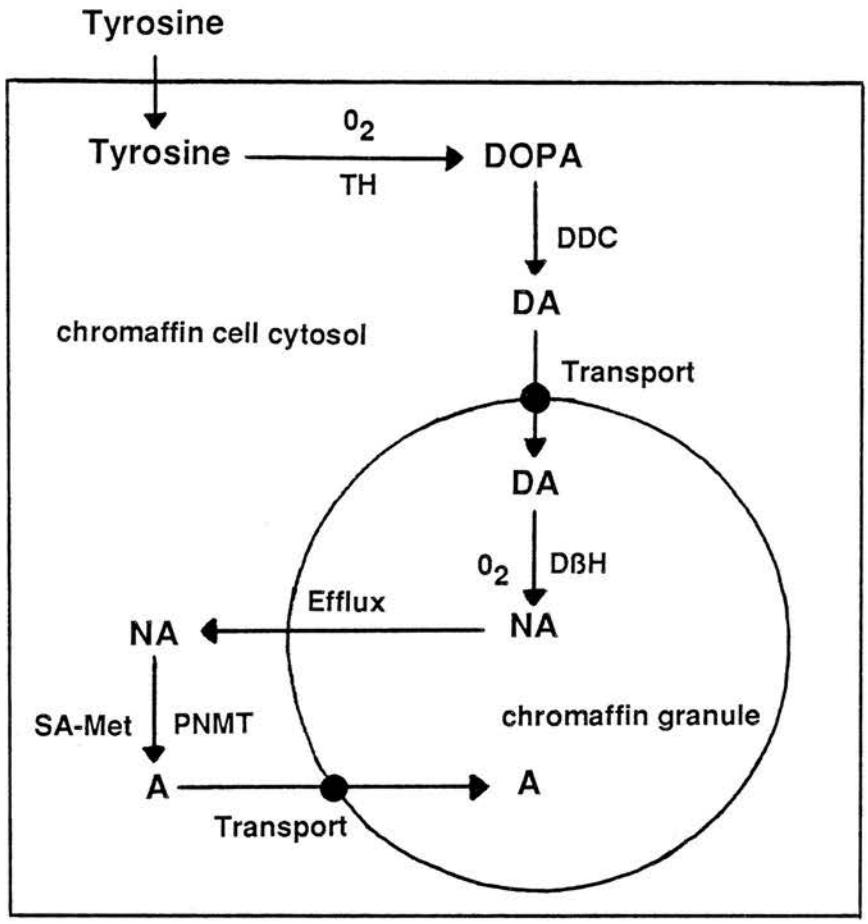



***Fig. 1.1 Biosynthesis of catecholamines in the adrenal medulla***

The abbreviations are: A, adrenaline; DA, dopamine; NA, noradrenaline; DOPA, 3,4-diydroxyphenylalanine; DβH, dopamine ß-hydroxylase; DDC, dopa decarboxylase; PNMT, phenylethanolamine *N*-methyltransferase; TH, tyrosine hydroxylase; SA-Met, *S*-adenosyl-L-methionine. For details see the text.

synthesis of catecholamines from the precursor amino acid, tyrosine. Many of these steps are integrally related to the chromaffin granule. Tyrosine is hydroxylated by the enzyme tyrosine 3-hydroxylase to form dopa, which, in turn is decarboxylated by dopa decarboxylase to dopamine (Fig.1.1). Tyrosine 3-hydroxylase is the rate-controlling enzyme of the biosynthetic pathway, its catalytic activity being subject to complex kinetic regulation by the cytosolic concentration of its substrates and co-factors. The activity of the enzyme is also increased by phosphorylation by several protein kinases in the cytosol, such as protein kinase C and $Ca^{2+}$/calmodulin dependent protein kinase (Vulliet 1984; Pocotte & Holz, 1986). Dopamine must accumulate within granules before it can be hydroxylated by dopamine ß-hydroxylase, an intragranular enzyme, to noradrenaline. In the adrenaline containing cells the noradrenaline leaks out of the storage granule and is methylated within the cytosol by the final enzyme in the biosynthetic pathway, phenylethanolamine N-methyl transferase (PNMT), found only in adrenaline containing cells. Finally, adrenaline is re-accumulated and stored within the granule (Fig. 1.1).

There are 20,000-30,000 chromaffin granules in a single chromaffin cell (Phillips, 1982) and the transport of catecholamines into the chromaffin granule may be summarised as serving the following functions : **1)** compartmentalization of the catecholamines away from the cytosol, thereby protecting them from degradative enzymes; **2)** biosynthesis of catecholamines, since dopamine ß-hydroxylase is present only within the matrix space; **3)** concentration and packaging of the amines against a large concentration gradient, thereby allowing large amounts of amines to be released by exocytosis; **4)** maintainance of the catecholamines in an environment where they will remain unoxidised, in the presence of an acid pH and the reductant ascorbate.

Points 3) and 4) will be discussed in more detail in later sections of this introduction.

### *1.1.3 Stimulus-Secretion Coupling in Chromaffin Cells*

The adrenal medulla, with its large population of catecholamine-containing chromaffin cells, provides one of the most convenient preparations in which to study the mechanism and regulation of secretion. The adrenal chromaffin cell is generally regarded as being very similar to a ganglion cell of the sympathetic nervous system, innervated by pre-ganglionic cholinergic neurones that arise in the intermediolateral cell column of the spinal cord. In mammals, most of these pre-ganglionic fibres pass directly via the thoracic splanchnic nerves to innervate the gland (Malmejac, 1964; Carmichael, 1987).

In virtually all species both nicotinic and muscarinic receptors are present on the chromaffin cell surface. In most species both types can evoke catecholamine secretion. However, in a few (e.g. chick) release is purely muscarinic, while in others (e.g. cow) only nicotinic receptors stimulate secretion. The major physiological stimulus for exocytosis is neurally released acetylcholine (ACh) acting on the chromaffin cell's nicotinic receptors. This causes the opening of the receptor's ion channel, which allows entry of sodium ($Na^+$) and, to a lesser extent, calcium ($Ca^{2+}$) ions. The result is a depolarisation of the chromaffin cell plasma membrane sufficient to activate voltage-sensitive ion channels (Corcoran & Kirshner, 1983; Kirshner 1986). The two main voltage-dependent channels are tetrodotoxin-sensitive $Na^+$ channels, which are highly selective for $Na^+$ ions, and slower dihydropyridine-sensitve $Ca^{2+}$ channels. The opening of both types of channels results in the firing of action potentials. However, the majority of the depolarisation that occurs is due to the tetrodotoxin-sensitive channels (Brandt *et al.,* 1976). A combination of receptor ion channel-induced depolarisation, and tetrodotoxin-sensitive action potentials further activates the voltage-dependent slow $Ca^{2+}$ channels. In unstimulated cells, the resting cytosolic free $Ca^{2+}$ concentration is of the order of 0.1$\mu$M or less. Activation of $Ca^{2+}$

channels allows the free $Ca^{2+}$ concentration to rise to 1μM or more, and this rise is the primary trigger for exocytosis of chromaffin granules containing catecholamine.

In recent years, a large number of studies have been addressed to the mechanism of $Ca^{2+}$-stimulated exocytosis from the adrenal chromaffin cells. The precise machinery, however, that transduces the cytosolic $Ca^{2+}$ signal into the mechanics of granule and plasma membrane fusion is still not clearly understood. The reader is referred to several important and influential reviews which cover this subject in some detail (Knight & Baker,1983; Burgoyne, 1984; Baker, 1988).

In the next section I will outline the salient features of this $Ca^{2+}$ stimulated exocytosis and show that the application of several powerful new techniques is rapidly establishing a molecular understanding of this process.

It is now widely recognised that an increase in the cytosolic free calcium concentration is a key step in the mechanism of secretion from cells such as chromaffin cells, as well as secretion from exocrine and neurotransmitter tissue. The adrenal chromaffin cell provides an excellent system for these studies as it has a well-characterised and easily monitored catecholamine secretory response which utilises an influx of extracellular calcium.

A very useful preparation for studying the mechanism of exocytosis is that of the "leaky" cell, first developed by Knight & Baker (1982). A series of brief pulses of high voltage create 10-20 pores in the plasma membrane, each with an effective diameter of 4nm. These pores allow the passage of molecules and ions of low molecular weight placed in the bathing solution, such as $Ca^{2+}$ and ATP; the movement of macromolecules is excluded. Low concentration of detergents such as digitonin (Dunn & Holz 1983; Wilson & Kirshner 1983) have also been used to permeabilise cells, but these generate larger holes and often have an inhibitory effect on exocytosis, perhaps because of leakage and consequently dilution of essential macromolecules. One of the main observations made with the Knight and Baker preparation is that secretion has an absolute requirement for MgATP, the half-maximal effect occurring at 1mM; ATP alone is ineffective and secretion does not occur when MgATP is replaced with a

variety of analogues. Using $Ca^{2+}$-EGTA solutions to buffer the free $Ca^{2+}$ concentration, less than 1% of the cellular catecholamine is released with a free $Ca^{2+}$ concentration of 10nM; when this is raised into the micromolar range, up to 30% of the cellular catecholamine appears in the external medium, with a threshold of about 0.5μM and half maximal effect being seen at about 1.0μM (Knight & Baker 1983). In these experiments, the release of catecholamines depends entirely on the free $Ca^{2+}$ to which the leaky cells are exposed, and the normal requirement for cholinergic stimulation is bypassed. The catecholamine release is exocytotic as it is accompanied by the release of granular dopamine ß-hydroxylase and the neuropeptide Met-enkephalin, but not by the cytosolic enzyme lactate dehydrogenase (Baker & Knight, 1981).

It is important to establish precisely the role of MgATP in exocytosis; this is necessary because of the very specific requirement of this substrate for catecholamine release in the presence of $Ca^{2+}$, and because ATP hydrolysis is required for accumulation and storage of catecholamines within the chromaffin granules themselves. The chromaffin granule membrane possesses an ATP-dependent proton pump, which maintains the interior of the granule at an acidic pH and electrically positive with respect to the cytosol (Percy *et al.,* 1985). Are these proton and pH gradients essential for exocytosis? In "leaky" cells the granule membrane pH gradient or potential were dissipated and the effect on $Ca^{2+}$-dependent release examined (Knight & Baker 1985). An inhibitor of the proton pump, trimethyl tin, was used to collapse the membrane potential and a proton ionophore, FCCP, was used to reverse it; $^{14}C$-labelled thiocyanate, a lipid-soluble anion, was used to monitor the membrane potential. These experiments showed that the $Ca^{2+}$-dependent release of catecholamine was unaffected by the absence of an inside-positive granule membrane potential. Similarly, 30mM ammonium sulphate ($(NH_4)_2SO_4$) was used to collapse the pH gradient across the membrane, as measured using [$^{14}C$]methylamine as a probe for intracellular compartments of acidic pH. As before, this intervention had no obvious effect on $Ca^{2+}$-dependent catecholamine release. These results therefore suggest that

ATP, although crucial for exocytosis, does not exert its effects via the granule proton pump.

### *1.1.4 Role of Intracellular Calcium in Secretion*

In recent years, with the development of a range of fluorescent $Ca^{2+}$-sensitive dyes, most notably Quin-2 and its superior successor Fura-2 (Tsien *et al.,* 1982; Grynkiewicz *et al.,* 1985 ), it has become possible to monitor free intracellular $Ca^{2+}$ concentrations directly. Using Quin-2, Kao & Schneider (1986) challenged intact (non-permeabilised) bovine chromaffin cells with acetylcholine (ACh) and derived a cytosolic $Ca^{2+}$ activation curve for catecholamine secretion, indicating that a threshold of about 0.25-0.3μM cytosolic $Ca^{2+}$ must be attained before detectable secretion occurs; half-maximal catecholamine release was achieved at about 2 μM $Ca^{2+}$, with maximal ACh-evoked release (about 25% of total cell catecholamine) occurring between 5 and 10 μM. These results were almost identical to those found using the "leaky" cell preparation of Knight & Baker (1982) and in digitonin permeabilised cells (Holz *et al.,* 1983; Wilson & Kirshner 1983). In addition, Kao & Schneider (1986) were able to measure the rate of decay of the maximal ACh-stimulated rise in cytosolic free $Ca^{2+}$. This seems to occur by at least two kinetically distinct processes with half-time decay constants of 0.6min for the first process and 3.2min for the second. The second process accounted for approximately 80% of the maximally stimulated rise in cytosolic free $Ca^{2+}$, and was postulated to utilise a high capacity, low affinity removal mechanism. The initial decay process may be a low capacity, high affinity mechanism.

As discussed by these authors, a variety of mechanisms probably interact to regulate cytosolic $Ca^{2+}$ following ACh receptor activation. These include receptor activation and desensitisation, and $Ca^{2+}$ channel activation and deactivation. However, the processes responsible for buffering cytosolic $Ca^{2+}$ and influencing their decay, are not understood. In a study using digitonin-permeabilised chromaffin cells, Kao

(1988) has investigated the role of intracellular organelles in regulating cytoplasmic $Ca^{2+}$ concentrations. The main findings were as follows: (1) that only about 10% of the $Ca^{2+}$ that enters the cell following stimulation with acetylcholine would be expected to be subsequently sequestered within subcellular organelles, with the remaining 90% presumably being extruded from the cell by the plasma membrane $Ca^{2+}$-ATPase and/or $Ca^{2+}/Na^{+}$ exchanger; (2) at low free $Ca^{2+}$ levels (0.1-0.2μM) most of the $Ca^{2+}$ uptake by cells was removed by an ATP-dependent mechanism into an uncoupler-insensitive compartment, probably the endoplasmic reticulum (ER); this conclusion was reached because the sequestration could be blocked by vanadate, an inhibitor of the $Ca^{2+}$-ATPase in the ER membrane, and was releasable by inositol trisphosphate ($IP_3$); (3) with micromolar free $Ca^{2+}$ concentrations (1μM and above), $Ca^{2+}$ uptake was also ATP-dependent, but uncoupler-sensitive, and it was concluded to have entered the mitochondria, since this uptake was almost completely abolished by azide, oligomycin and 2,4-dinitrophenol (DNP). Kao (1988) concluded that chromaffin granules were unlikely to play a significant role in $Ca^{2+}$ homeostasis in these permeabilised chromaffin cells.

However, in other studies using Quin2, the level of $[Ca^{2+}]_i$ detected in chromaffin cells stimulated maximally with cholinergic agonists, such as carbamylcholine and acetylcholine, is of the order of 0.3-0.5μM (Burgoyne, 1984; Burgoyne & Cheek, 1985) values that are much lower than those found by Kao & Schneider (1986). Similar, or even slightly higher values were found in cells depolarised with high concentrations of external $K^{+}$ (55mM), or after treatment with the ionophore A23187 (Burgoyne, 1984), even though these agents elicit much less secretion than do cholinergic agonists.

Cobbold *et al.,* (1987), using single isolated chromaffin cells microinjected with the intracellular $Ca^{2+}$ probe aequorin, showed that 55mM $K^{+}$ or 10μM nicotine resulted in only a transient (60-90sec) elevation of free $Ca^{2+}$; the maximal peak rise in free $Ca^{2+}$ was up to 1μM, and was the same using either agent to elicit the response. These results are in contrast to the Quin2 measurements made on cell

populations, in which a sustained rise in $[Ca^{2+}]_i$ is seen. The difference may be due to significant buffering of cytosolic $Ca^{2+}$ by Quin2, which is present at up to 1mM; this may mask the transient nature of the response by raising the quantity of $Ca^{2+}$ that has to be removed to restore resting free $Ca^{2+}$ levels. Of course, a nicotine-stimulated rise in free $[Ca^{2+}]$ to 0.3-0.5μM may itself be insufficient to initiate a full secretory response; it is likely, therefore, that an additional (but as yet unidentified) second messenger capable of mobilising $Ca^{2+}$ from an intracellular store is required (Cobbold *et al.,* 1987).

### *1.1.5 Role of Contractile Proteins in Secretion*

Early studies (e.g. Poisner & Cooke, 1975) provided some evidence that the movement of chromaffin granules from the Golgi region to the plasma membrane involved some form of "directed" transport, rather than simple random movement. These authors showed that chromaffin cells contained an abundance of microtubules, mostly arranged radially with respect to the plasma membrane. The microtubules could be disrupted by treatment with colchicine or vinblastine, which also inhibits acetylcholine-stimulated catecholamine release from perfused adrenal glands (Poisner & Bernstein, 1971). However, colchicine does not block secretion induced by high $K^+$ in intact cultured cells (McKay & Schneider, 1984), or $Ca^{2+}$-induced secretion in electropermeabilised cells (Baker & Knight, 1981). It is still not clear, therefore, whether microtubules are components essential for exocytosis.

A large number of cytoskeletal proteins are present in adrenal chromaffin cells and recent work has provided considerable support for the hypothesis that $Ca^{2+}$ releases chromaffin granules from a cytoskeletal network. Chromaffin cells contain myosin, which seems to be mostly attached to the plasma membrane (Trifaro *et al.,* 1978). Actin is also present mainly in the cytosol but also associated with the granule membranes (Phillips & Slater,1975; Aunis *et al.,* 1980). F-actin interacts with the

granule membrane in a $Ca^{2+}$-dependent manner, binding sites for F-actin being provided by molecules of α-fodrin (Fowler & Pollard, 1982; Kao & Westhead, 1984).

Extracts of cytoplasm from adrenal chromaffin cells form, *in vitro*, a "supramolecular gel" containing cytoskeletal elements; these are microfilaments, microtubules and intermediate filament proteins. The latter have been identified as filamin, fodrin, caldesmon, myosin and tropomyosin (Burgoyne *et al.,* 1986; Burgoyne & Cheek, 1987). How do these proteins fit with a coherent picture of exocytosis? It seems likely that in resting (unstimulated) cells exocytosis is prevented by the immobilisation of granules in a cytoskeletal network (Lelkes *et al.,* 1986; Burgoyne & Cheek, 1987). Following $Ca^{2+}$ entry though the plasma membrane, the network is disrupted and the granules are free to fuse with the plasma membrane. A number of experimental observations support this idea. α-Fodrin, an actin binding protein, is found as a continuous ring beneath the plasma membrane (as shown by immunofluorescence microscopy). After stimulation of cells using 10μM nicotine or 56mM $K^+$, fodrin distribution was more patchy, with, respectively, 95% and 60% of cells displaying this patchy appearance after 30min (Perrin & Aunis, 1985).

During stimulation with nicotine (although not with high $K^+$) actin filaments are first disassembled and then reassembled (Cheek & Burgoyne, 1986). Disassembly of actin in a $Ca^{2+}$-dependent manner by a gelsolin-like protein has been demonstrated in bovine chromaffin cells (Bader *et al.*, 1986). Thus, a more integrated picture of secretion, involving a dynamic interaction between secretory and plasma membranes with the cytoskeletal network, is beginning to emerge. However, several important aspects remain unsolved. These include the effect of guanine-nucleotide binding proteins (G proteins) on the cytoskeleton (Burgoyne, 1987) and the nature of events involved in the plasma and secretory granule membrane fusion, which is still almost a complete mystery (Linstedt & Kelly1988).

### *1.1.6 Protein Phosphorylation in Secretion*

A possible explanation for the specific involvement of MgATP in exocytosis is the control by phosphorylation of certain key proteins. Stimulation with secretagogues (reagents that initiate secretion) results in the increased incorporation of $^{32}P$ into certain proteins prior to the onset of catecholamine release. However, it is vital to determine the role of phosphorylated proteins in particular subcellular compartments, as minor, but still important, phosphoproteins may be missed in examinations of the whole cell. To this end, protein phosphorylation has been examined in the chromaffin granule membrane, and a number of protein kinases which are stimulated by cyclic AMP or calmodulin have been found (Burgoyne & Geisow, 1981; Treiman *et al.,* 1983). For example, three polypeptides of molecular weight 59, 58 and 53 kDa, which are substrates for a calmodulin- dependent protein kinase activated half maximally at about 5μM $Ca^{2+}$, have been reported (Burgoyne & Geisow, 1981). The 59 kDa protein is tyrosine hydroxylase, the rate-controlling enzyme of catecholamine biosynthesis, but the roles of others are unknown, although all are on the cytoplasmic surface of the granules. Phosphorylation of tyrosine hydroxylase activates the enzyme presumably to replenish catecholamine stores following exocytosis.

Dephosphorylation reactions may also be important during secretion. The ATP analogue adenosine 5'-O-(3-thiotriphosphate) is a substrate for protein kinases and the thiophosphoprotein that is formed is resistant to phosphatase-catalysed dephosphorylation. Because this thio-analogue competed with MgATP and irreversibly inhibited secretion, the suggestion has been made that dephosphorylation could be rate-determining in exocytosis (Brooks *et al.,* 1984).

### *1.1.7 Protein Kinase C*

MgATP may also be a substrate for the enzyme protein kinase C, which is activated by $Ca^{2+}$ and diacylglycerol. In electropermeabilised ("leaky") cells, the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) shifts the $Ca^{2+}$ activation curve for catecholamine release to the left, i.e. to lower $Ca^{2+}$ concentrations (half-maximal activation at approximately 0.5μM, compared with 1.0μM in the absence of TPA). Other reagents which do not activate protein kinase C, such as phorbol and phorbol-13-acetate, were ineffective (Knight & Baker, 1983). The attraction of protein kinase C as a key intermediate in stimulus-secretion coupling is that one single molecule serves as a receptor for $Ca^{2+}$, diacylglycerol and ATP. Moreover, protein kinase C binds to the chromaffin granule membranes in a $Ca^{2+}$-dependent fashion (see below) and exocytosis from the "leaky" adrenal cells is blocked by trifluoperazine, which, as well as being an inhibitor of calmodulin, is also a potent inhibitor of protein kinase C (Scott-Turner *et al.,* 1982) .

Diacylglycerol (DAG), which is an activator of protein kinase C, is produced in many plasma membranes as a result of activation of phospholipase C (PLC) coupled to a direct receptor-mediated mechanism, or by $Ca^{2+}$ influx. In chromaffin cells, a rise in cytosolic $Ca^{2+}$ induced by nicotinic agonists or 56mM $K^+$ directly stimulates PLC (Eberhard & Holz, 1987); this activation is dependent upon extracellular $Ca^{2+}$ and is inhibited by the calcium channel antagonists D600 and nifedipine. In contrast, muscarinic agents (which do not cause secretion from bovine cells) also activated PLC through a receptor-mediated mechanism that was independent of external $Ca^{2+}$. Therefore, in the same cells, the mechanism of PLC activation depends on the type of signal at the plasma membrane.

The other product of PLC action, *myo*-inositol 1,4,5-trisphosphate ($IP_3$), stimulates the release of $Ca^{2+}$ from intracellular compartments in numerous cell types (see Berridge, 1987 for a review). $IP_3$ has been shown to cause $Ca^{2+}$ release

from an unknown intracellular store into the cytosol of permeabilised bovine chromaffin cells (Stoehr *et al.*, 1986), although the amount released was insufficient to stimulate catecholamine secretion. It has not yet been proved that the intracellular $Ca^{2+}$ store is the ER, and to date no studies on $IP_3$ -induced $Ca^{2+}$ release from isolated adrenal chromaffin cell ER preparations have been reported.

### *1.1.8 Calcium-Dependent Interaction of Chromaffin Granules with Cytosolic Proteins*

#### *1.1.8.1 Calmodulin*

Calmodulin is a ubiquitous $Ca^{2+}$-binding protein that is present in the adrenal medulla at an intracellular concentration of about 1μM (Kuo & Coffee,1976). Hikita *et al.,* (1984) have made a thorough investigation of the properties of chromaffin cell calmodulin, its subcellular distribution, and its interaction with the chromaffin granule membrane. Their study of the binding of $^{125}$I-calmodulin to granule membranes showed that in the presence of 100μM free $Ca^{2+}$, saturable and high affinity calmodulin-binding sites (dissociation constant ($K_D$) = 9.8nM; maximal binding capacity ($B_{max}$) = 25pmol.mg$^{-1}$ membrane protein) were present. In addition, a non-saturable calmodulin-binding site was detected between free $Ca^{2+}$ concentrations of 0.1 and 0.25μM; this latter binding of $Ca^{2+}$ was lost, however, following delipidation of the membranes. These workers hypothesised that between 0.1 and 0.25μM free $Ca^{2+}$ one or two molecules of $Ca^{2+}$ are bound per calmodulin molecule, resulting in the exposure of a hydrophobic surface, which may explain the binding of calmodulin to some hydrophobic components of the granule membrane (Bader *et al.,* 1985).

In a similar study, however, Burgoyne & Geisow (1981) obtained a rather different value for binding ($K_D$ = 31nM and $B_{max}$ = 3.3 pmol.mg$^{-1}$membrane

protein) with sites of lower affinity ($K_D$>100nM) also present. Given an intracellular calmodulin concentration of 1μM these low affinity sites could still be of physiological significance. Bader *et al.,* (1985) identified two calmodulin-binding proteins, of molecular weights 65 and 53 kDa, in the granule membrane and proposed that the former may play a role in the interaction between calmodulin and the secretory granule. Using monoclonal antibodies the 65kDa calmodulin-binding protein has also been found in neurohypophyseal and synaptic vesicles, as well in chromaffin granules. (Bader *et al.,* 1985; Fournier & Trifaro, 1988a). In their most recent work, Fournier & Trifaro (1988b) have demonstrated binding of the 65 kDa protein to the chromaffin cell plasma membrane. These results suggest that this and other calmodulin-binding proteins may be important in directing the fusion between plasma and secretory vesicle membranes during exocytosis; thus, their role as "docking sites" in membrane interactions, although not proven, is an attractive hypothesis.

#### *1.1.8.2 Synexin*

In 1978, the first example of a chromaffin cell cytosol protein that showed a $Ca^{2+}$- dependent interaction with granules was purified, and was called "synexin", because of its ability to aggregate isolated chromaffin granules in the presence of $Ca^{2+}$ (Creutz *et al.,* 1978). Synexin is a 47kDa protein, and Pollard and his colleagues (Pollard *et al.,* 1986) have proposed a novel "hydrophobic bridge" hypothesis for its action: synexin molecules, in the presence of $Ca^{2+}$, form hydrophobic polymers capable of binding to specific membranes and inserting into the bilayer; subsequently, the synexin polymer would penetrate into the bilayer of juxtaposed membranes causing them to adhere. This latter event is envisaged as a necessary step towards eventual fusion. However granule-granule fusion is not an ideal system for the study of exocytosis since this sort of interaction does not occur in the intact cell. Furthermore, the affinity of synexin for $Ca^{2+}$ is very low ($K_D$= 200 μM); therefore,

it seems unlikely that synexin is involved in secretion since exocytosis is triggered half-maximally at an intracellular free $Ca^{2+}$ concentration of 1μM.

### *1.1.8.3 Chromobindins - A Family of Calcium-Dependent Granule Binding Proteins*

During the 1980s, a bewildering number of proteins which associate reversibly with biological membranes in the presence of micromolar concentrations of $Ca^{2+}$ have been identified in a number of different tissues. Geisow & Burgoyne (1982) and Creutz *et al.,* (1983) purified a large number of proteins from adrenal chromaffin cell cytosol by $Ca^{2+}$-dependent affinity chromatography, and these proteins were named "chromobindins" by Creutz. A similar approach was used with the electric organ of the ray *Torpedo marmorata* (Walker, 1982) and yielded a protein that has been called calelectrin. At least 23 "chromobindins" are known to bind to chromaffin granule membranes *in vitro* in the presence of $Ca^{2+}$, and these include several well known proteins already described, such as calmodulin, protein kinase C and synexin. These proteins have been the focus of considerable attention since their discovery as possible mediators of a number of intracellular events in the exocytosis/endocytosis cycle.

Creutz *et al.,* (1987) have summarised currently understood characteristics of the chromaffin granule binding proteins and a table of these characteristics from their paper is reproduced in Table 1.1. However, as Creutz *et al.,* (1987) have pointed out, these proteins are probably similar to, if not identical with proteins already purified from other tissues. Thus, calelectrin from *Torpedo* has been identified as chromobindin 4, and this is probably similar to another protein found in mammalian liver. Calcimedin from smooth muscle (known as p35) is a substrate for the tyrosine kinase activity associated with the epidermal growth factor (EGF) receptor, and this is probably the same as chromobindin 6 and several plasma membrane binding proteins from lymphocytes. Creutz *et al.,* (1987) have

*TABLE 1.1 Characteristics of the $Ca^{2+}$-dependent chromaffin granule membrane binding proteins**

| Chromobindin number | Mol. Wt (kDa) | Isoelectric point (pI) | ATP | Identity |
|---|---|---|---|---|
| 1 | 19 | | - | Calmodulin |
| 2 | 26 | 5.7 | - | Unknown |
| 3 | 26 | 6.8 | - | Unknown |
| 4 | 32 | 5.8 | - | 32kDa Calelectrin |
| 5 | 32.5 | 5.2 | - | Unknown |
| 6 | 33 | 6.7 | - | Fragment of Chromobindin 9 |
| 7 | 35 | 5.1 | - | Unknown |
| 8 | 36 | 7.9 | - | p36 |
| 9 | 37 | 6.9 | - | EGF receptor substate (or calpactin II) |
| 10 | 46 | 5.6 | - | Actin |
| 11 | 47 | 7.0 | - | Synexin |
| 12 | 53 | 6.8 | + | Subunit of Chromobindin A |
| 13 | 55 | 7.8 | + | Subunit of Chromobindin A. |
| 14 | 56 | 5.9 | + | Subunit of Chromobindin A. |
| 15 | 56.5 | 6.2 | + | Subunit of Chromobindin A. |
| 16 | 57 | 7.0 | + | Subunit of Chromobindin A. |
| 17 | 57.5 | 5.7 | - | Unknown |
| 18 | 58 | 6.0 | + | Subunit of Chromobindin A. |
| 19 | 59 | 7.0 | + | Subunit of Chromobindin A. |
| 20 | 67 | 6.1 | - | 67kDa calelectrin (synhibin) |
| 21 | 68 | 5.6 | - | Unknown |
| 22 | 83 | | | Protein Kinase C |
| 23 | - | | | Phosphatidylinositol Phospholipase C. |

* modified from Creutz *et al.*, (1988).

+ denotes that ATP is required for binding of chromobindin to the granule membrane;

- denotes that ATP is not required for binding.

demonstrated that chromobindin 8 (also known as p36, or calpactin heavy chain) undergoes phosphorylation during exocytosis, and since it has recently been shown that protein kinase C (chromobindin 22) phosphorylates p36 on serine residues (Gould *et al.,* 1986; Khanna *et al.,* 1986), protein kinase C becomes a strong candidate for mediating the phoshorylation of chromobindin 8 during secretion. Moreover, p36, together with two proteins of molecular weight 10kDa (calpactin light chains) constitute the calpactin tetramer which has the ability to promote granule membrane fusion with micromolar concentrations of $Ca^{2+}$; arachidonic acid is also required for membrane fusion and Creutz *et al.,* (1988) postulate that this is necessary for the reduction of surface charge before efficient membrane fusion can occur.

Seven of the "chromobindins" (Nos. 12-16, 18 and 19) are involved in forming a large macromolecular complex called chromobindin A. Interestingly, ATP is not required to promote binding of the subunits to the membrane, but is needed to permit release of the protein from the membrane in the absence of $Ca^{2+}$. The isolated complex has a slow ATP-hydrolysing activity, with a low specific activity (about 20 $nmoles.mg^{-1}.min^{-1}$) and a $K_m$ for ATP of 115$\mu$M, which correlates well with the estimate of the ATP concentration that causes half maximal release of chromobindin A from the membrane (Martin & Creutz, 1987).

The function of chromobindin A in the cell is not known. An attractive hypothesis is that it may be involved in the movement of granules along the cytoskeleton prior to membrane fusion, a role that has recently been attributed to kinesins in the brain (Vale *et al.*, 1986,1987).

## *1.2 The Chromaffin Granule - Structure and Dynamics*

### *1.2.1 Advantages in Studying the Chromaffin Granule as a Model Neuroendocrine Storage Organelle*

There are several reasons why the chromaffin granule has become the preferred neuroendocrine secretory granule for the studies of structure, function and composition; these may be listed as follows :

**(a)** large numbers of adrenal glands can be obtained from slaughterhouse animals such as pigs and cows, and the chromaffin cells within the medulla yield a large number of chromaffin granules;

**(b)** it is easy to isolate granules rapidly using differential and isotonic density gradient centrifugation because of the high density of the granules (1.18 $g.cm^{-3}$, equivalent to 1.8M sucrose);

**(c)** the high density of the granules allows purification to a very high degree, the granules being largely devoid of contaminating subcellular organelles such as mitochondria, and other fragmented membranes.

**(d)** purified chromaffin granules may be subjected to hypo-osmotic shock to yield resealed "ghosts" which lack endogenous ion gradients and intragranular matrix components. Resealed "ghosts" are extremely useful for the study of membrane transport properties as the composition of their internal matrix can be varied at will, thus allowing transmembrane concentration gradients to be easily calculated.

Because cells of the adrenal medulla and sympathetic nervous system have a common embryological origin, it is often assumed that they have similar mechanisms for the synthesis, storage and release of catecholamines and neurotransmitters; Therefore, studies of these phenomena in the secretory organelles of chromaffin cells may be extrapolated to less readily isolated granules from other secretory cells. These include the 5-hydroxytryptamine-containing dense granules of platelets, histamine granules of mast cells, insulin granules from the ß-cells of the pancreas, and the

acetylcholine-containing cholinergic vesicles of the marine ray, *Torpedo*.

### *1.2.2 Composition of the Chromaffin Granule Membrane and Matrix*

A comprehensive overview of the composition of the chromaffin granule is shown in Table 1.2 Most of the information in this table has been taken from reviews by Winkler (1976), Winkler & Carmichael (1982) and Winkler & Westhead (1980).

### *1.2.3 Membrane Proteins and Lipids*

Chromaffin granule membranes contain about 20% of granule protein. They are characterised by a high lipid-to-protein ratio (approx. 2μmol phospholipid per mg of protein), and, within the lipids, high contents of cholesterol (0.6μmol/μmol phospholipid) and lysophosphatidylcholine (18% of lipid phosphorous). Adherent matrix proteins can be removed from membranes by washing or centrifugation, although it is often hard to know whether cytoplasmically-oriented proteins are peripheral membrane proteins or cytoplasmic contaminants. Following two-dimensional sodium dodecyl sulphate (SDS) gel electrophoresis and staining with Coomassie Blue, more than 40 polypeptides are resolved (Abbs & Phillips 1980). However, only a few of the proteins identified from two-dimensional gels have been assigned a particular function, and only those constituting the largest proportion have been purified to homogeneity. These include the proton-translocating adenosine triphosphatase ($H^+$-ATPase), cytochrome $b_{561}$ and dopamine ß-hydroxylase (DßH), and will be considered in more detail in the section describing the bioenergetics and transport properties of the granule membrane.

Many of the proteins of the granule membrane are glycoproteins, and can be revealed following SDS-gel electrophoresis, transfer onto nitrocellulose sheets, and decoration with various lectins. Gavine *et al.*, (1984) and Pryde & Phillips (1986) have carried out a thorough analysis of chromaffin granule membrane glycoproteins;

## *Table 1.2 Composition of Bovine Chromaffin Granules*

| ***Membrane Proteins*** | ***appox. Mol.Wt. (kDa)*** |
|---|---|
| $Mg^{2+}$ ATPase I (Proton-translocating) | 400 |
| $Mg^{2+}$ ATPase II (Unknown function) | 140 |
| Cytochrome $b_{561}$ | 120 |
| Dopamine ß-Hydroxylase | 290 (tetrameric) |
| Phosphatidylinositol Kinase | ? |
| Catecholamine Transporter | ? |
| $Ca^{2+}/Na^{+}$ Exchanger | ? |
| Glycoproteins (II, III, J, K) | ? |
| Synaptin | ? |
| Synaptophysin | 38 |

| ***Membrane Lipids*** | ***nmol.mg$^{-1}$ membrane protein*** |
|---|---|
| Cholesterol | 1440 |
| Phosphatidylcholine | 650 |
| Lysophosphatidylcholine | 410 |
| Sphingomyelin | 290 |
| Phosphatidylethanolamine | 840 |
| Phosphatidylinositol | 200 |
| Phosphatidylserine | 60 |
| Ganglioside $GM_1$ | 50 |
| Fatty Acids | 100 |

| ***Glycosaminoglycans*** | ***nmol sugar.mg$^{-1}$ membrane protein**** |
|---|---|
| Chondroitin Sulphate | 3000 |
| Heparin Sulphate | 400 |

* data from Geissler *et al.*, (1977)

| ***Matrix Proteins and Peptides*** | |
|---|---|
| Chromogranin A Family | |
| Chromogranin B Family | |
| Chromogranin C Family | approx. 4mg/mg membrane protein |
| Proenkephalin Family | approx. 180mg.ml$^{-1}$ |
| Acetylcholinesterase | (Chromogranin A family about 50% of total) |
| Proteases | |
| Dopamine ß-Hydroxylase | |

| ***Matrix Glycosaminoglycans*** | ***nmol sugar.mg$^{-1}$ membrane protein**** |
|---|---|
| Chondroitin Sulphate | 2600 |
| Heparin Sulphate | 100 |

| ***Matrix Small Molecules*** | ***nmol.mg$^{-1}$ granule protein*** | ***mM*** ** |
|---|---|---|
| Adrenaline | 1800 | |
| Noradrenaline | 680 | 550 |
| Dopamine | 20 | |
| ATP | 560 | 130 |
| GTP | 76 | 18 |
| UTP | 43 | 10 |
| ADP | 66 | 15 |
| AMP | 38 | 9 |
| Ascorbic Acid | 100 | 23 |
| Enkephalins | 7 | 1.6 |
| $Ca^{2+}$ | 90 | 20 |
| $Mg^{2+}$ | 20 | 5 |
| $Na^{+}$ | 94 | 22 |

* data from Geissler *et al.*, (1977); ** calculated assuming an internal volume for granules of 4.3µl.mg$^{-1}$ protein (Pollard *et al.*, (1976); Phillips *et al.*, (1977)).

these studies suggest that the membranes contain about 20 glycoproteins and that their sugar residues are matrix oriented. The function of the majority of the glycoproteins is unknown.

### *1.2.4 Matrix Proteins and Peptides*

The total protein content of the chromaffin granule matrix is about 180mg.ml$^{-1}$, a remarkably high figure, and most of this is accounted for by a series of three 45 to 70 kDa proteins (Chromogranins A, B and C). Chromogranin A is the major one, making up 50% of the total matrix protein; it is very acidic with an isoelectric point of about 4·8. It is a glycoprotein, with galactose, N-acetylglucosamine and sialic acid as the major sugars. Chromogranin A consists of 431 amino acids with a high content of glutamic acid and proline residues and five phosphoserine residues. In 1982, a protein isolated from parathyroid secretory granules, called secretory protein I was shown to be very similar to chromogranin A, and wide occurrence of the three different type (Chromogranins A, B, and C) has now been reported in numerous endocrine and neural tissues (see review by Winkler *et al.,* 1986). Chromogranin A from bovine adrenal medullary cells was the first to be cloned and sequenced (Iacangelo *et al.,* 1986; Benedum *et al.,* 1986) and has an actual molecular mass of 48 kDa (compared with its apparent molecular mass of 70-77 kDa from SDS PAGE). Human chromogranin B has also been sequenced (Benedum *et al.*, 1987). It has some homology with chromogranin A, and has a similar isoelectric point, but a molecular mass of 76 kDa.

The function of the chromogranins is still unknown, although several possibilities have been advanced. Matrix chromogranin A (and possibly chromogranins B and C) is responsible for the binding of much of the 20mM or so total $Ca^{2+}$ present within the matrix (Reiffen & Gratzl, 1986); it has also been postulated to be a sorting element involved in segregation of proteins into the secretory pathway (Benedum *et al.*, 1986). NMR studies have shown that the bulk of the matrix chromogranin A is

random coil and is in rapid motion (Sharp & Richards, 1977; Sen & Sharp, 1980, 1981) and probably does not contribute greatly to catecholamine binding within the matrix (Winkler & Westhead, 1980).

There are two minor groups of matrix proteins : glycoproteins and enkephalin precursors. Of the glycoproteins, dopamine ß-hydroxylase also has a membrane-associated form and, in bovine granules, approximately 50% of the enzyme is membrane-bound and 50% is within the matrix (Winkler *et al.*, 1986). Enkephalins are also stored within the granule matrix, where processing enzymes (proteases) degrade the precursor proenkephalin A into Met- and Leu-enkephalins in the ratio of 5:1 (see Winkler *et al.*, 1986, for a review). Like the chromogranins, no definite function of the adrenal enkephalins has been established. Finally, a host of small peptides are present : neuropeptide Y, bombesin, vasoactive intestinal peptide and dynorphin.

Acetylcholinesterase has been reported to be present both in the matrix and as a membrane-bound form (Gratzl, 1984), although it is possible that some of this may originate from chromaffin cell plasma membranes or from the endoplasmic reticulum (Mizobe *et al.*,1984).

### *1.2.5 Bioenergetics and Membrane Transport Properties of Chromaffin Granule Membranes*

In 1961, Peter Mitchell revolutionised our understanding of biological energy processes when he proposed the chemiosmotic hypothesis to explain how scalar energy forces could result in the vectorial transport of solutes across membranes. The basic assumptions of this hypothesis have remained intact since then, and it is worth summarising them here. First, a topographically closed insulating membrane is required, with a very low permeability to ions and solutes. Second, a vectorially oriented proton-translocating adenosine triphosphatase ($H^+$-ATPase) within the membrane is used to generate, or to utilise, an electrochemical proton gradient. Third, the proton electrochemical potential may be coupled to ion or metabolite transport using proton-linked transport systems within the membrane. The chromaffin granule exemplifies this basic model.

It is well-established that the chromaffin granule has a very low permeability to ions such as $H^+$, $Ca^{2+}$, $Mg^{2+}$, $Na^+$ and $K^+$; in particular, $H^+$ conductance across the membrane is extremely low (0.088μg/sec/pH unit per gram), which is about one order of magnitude less than that across the inner mitochondrial membrane (Johnson & Scarpa, 1976b). Experiments investigating the permeability of isolated chromaffin granules to small ions have included : light scattering of granules as a function of osmolarity; light scattering of granules in isotonic solutions of various inorganic salts; swelling of chromaffin granules induced by various ionophores; and direct measurements of $H^+$ uptake or release induced by various ionophores (Johnson & Scarpa, 1976b). It was measurements of the low proton permeability across the chromaffin granule membrane that prompted the idea that a pH gradient (transmembrane $H^+$ concentration gradient, or $\Delta pH$) and perhaps a membrane potential (transmembrane electrical potential gradient, or $\Delta\Psi$) existed across the membrane. Using a number of different methods, such as measurements of

intragranular buffering capacity, permeant weak base accumulation, and $^{31}P$ nuclear magnetic resonance (NMR) spectroscopy, the intragranular pH of isolated chromaffin granules has been confirmed by numerous investigators to be between 5.5 and 5.7 (Johnson & Scarpa, 1976b; Bashford *et al.,* 1976; Casey *et al.,* 1977). Similar or slightly lower values are probably found in intact tissues (Bevington *et al.*, 1984).

The transmembrane electrical potential across the chromaffin granule membrane can be measured by monitoring the distribution of lipophilic ions such as thiocyanate ($SCN^-$) and trimethylphenyl phosphonium ($TPMP^+$); these ions distribute according to the membrane potential and give a measurement of potential, either positive or negative inside, respectively (Johnson & Scarpa, 1979; Salama *et al.,* 1979). With this knowledge, the transmembrane electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$ in mV) across the membrane can be calculated:

$$\Delta\tilde{\mu}_{H+} = \Delta\psi - (2.3RT/F)\Delta pH \qquad (mV) \qquad (1)$$

where R is a constant, T is the absolute temperature and F is the Faraday constant.

It has now been observed that in the absence of MgATP, the resting membrane potential of freshly-isolated chromaffin granules is about -10 to -20mV; addition of MgATP reverses this and a new steady value of about +60mV is attained. Therefore, the chromaffin granule, with its acidic matrix of pH 5.5 and $\Delta\Psi$ of about 80mV in the presence of MgATP at physiological pH, can generate an electrochemical potential gradient of protons of about 200 mV *in vitro* (eqn. 1). This electrochemical gradient is generated and maintained *in vivo* by the $H^+$-ATPase in the granule membrane which hydrolyses cytosolic (extragranular) ATP and catalyses inwardly-directed electrogenic proton translocation.

### *1.2.6 Proton-Translocating ATPase*

In recent years, much new information has become available regarding the

structure and characteristics of $H^+$-ATPases (or proton pumps). The enzyme present in the chromaffin granule that is responsible for the generation of the proton electrochemical gradient has been called ATPase I by Percy *et al.,* (1985); the enzyme is of the vacuolar (V) type, as similar types of ATPases are found in organelles such as lysosomes and endosomes (in animal cells), tonoplasts (in plant and fungal cells), and in other secretory vesicles such as the cholinergic synaptic vesicles from *Torpedo*, platelet dense vesicles and insulin secretory vesicles (Rudnick, 1986; Mellman *et al.,* 1986).

Purified chromaffin granule membranes do contain small amounts of $F_1F_0$ ATPase subunits derived from contaminating mitochondria, although these are entirely separate from the proton pumping activity of the endogenous enzyme (Cidon & Nelson, 1983). As with the other vacuolar ATPases, the chromaffin granule $H^+$ pump is inhibited by N,N'-dicyclohexylcarbodiimide (DCCD), alkylating reagents (such as N-ethylmaleimide), trialkyl tin and quercetin. It is not inhibited by vanadate, and inosine 5'-triphosphate (ITP) as well as ATP can serve as a substrate, albeit not as effectively (Percy *et al.,* 1985).

### *1.2.7 Catalytic Mechanism of ATPase I*

The enzyme works by hydrolysing cytosolic (extragranular) ATP and pumping protons into the matrix, resulting in intravesicular acidification. If a permeant anion such as chloride ($Cl^-$) or iodide ($I^-$) is present in the external medium, these will enter the matrix by passive diffusion to compensate for the electrogenic proton influx. However, high concentrations of some anions, such as nitrate, inhibit the enzyme significantly (Moriyama & Nelson, 1987). The stoichiometry of the enzyme activity (i.e. the number of $H^+$ translocated per ATP hydrolysed) has been measured under carefully controlled conditions, with values from 1.52 to 1.75 being obtained (Johnson *et al.,* 1982a); this may be an underestimate as some of the ATP may be hydrolysed without concomitant $H^+$ translocation (see below).

The subunit structure and topography of ATPase I is still a matter of debate. According to Apps & Percy (1986) there are at least five subunits, of molecular weight 70, 57, 40, 33 and 16 kDa (by SDS-PAGE), which can be separated following fractionation of the chromaffin granule membranes with the detergent Triton X-114. The small (16 kDa) subunit is reported to be part of the hydrophobic proton channel within the membrane which can be labelled by DCCD, and consists of 155 amino acid residues (Mandel *et al.*, 1988). DCCD is proposed to bind to a glutamic acid residue in one of the four transmembrane segments that spans the membrane. The 70 kDa subunit can be labelled with low concentrations of [$^3$H]NEM, and, as this labelling is significantly reduced in the presence of ATP, it was concluded that this is the catalytic subunit (Apps & Percy, 1986). Another group has demonstrated the presence of four subunits of molecular weight 115, 72, 57 and 39kDa, with [$^3$H] NEM labelling of the 115kDa subunit and [$^{14}$C]DCCD labelling of the 115 and 39kDa subunits (Cidon & Nelson, 1986). Thus, the two preparations seem to have at least three polypeptides in common (70 - 72, 57 and 39 - 41kDa). However, no function has yet been ascribed to the 57 and 39 - 41kDa polypeptides.

In addition to ATPase I (the proton pump), another ATPase in the chromaffin granule membrane has been described by Apps *et al.,* (1983), Cidon and Nelson (1983) and Percy *et al.,* (1985), and in fact accounts for 30 to 50% of the total membrane ATPase activity. It has been termed ATPase II, and has an apparent molecular weight of 115kDa. Its inhibitor sensitivity is different from that of the ATPase I, and it is not really affected by NEM, DCCD, trialkyl tin or quercetin, although it is potently inhibited by vanadate. It is unlikely to be a $H^+$-translocating ATPase and in fact its nature remains as an enigma. Taken together, the two ATP hydrolytic activities complicate inhibitor studies and measurements of $H^+$: ATP stoichiometry. However, the rate of ATP hydrolysis and $H^+$-translocation by ATPase 1 demonstrate simple Michaelis-Menten kinetics : the apparent $K_m$ for MgATP is

69μM and that for CaATP 140μM (Johnson *et al.,* 1982a ; Flatmark *et al.,* 1985). As the cytosolic ATP concentration is in the low mM range, these values suggest that the ATPase activity in the intact cell is not influenced by changes in cytosolic ATP levels.

### *1.2.8 Catecholamine Transport*

In chromaffin granules, the matrix catecholamine (CA) concentration (0.55M, see Table 1.2) is at least $10^4$ times the cytosolic concentration, and accumulation occurs by means of a specific reserpine-sensitive carrier which catalyses proton/amine exchange with a net stoichiometry of $2H^+$ per protonated amine (Johnson & Scarpa, 1979). Actually, it is hard to distinguish between this stoichiometry and that in which one proton is exchanged for a neutral catecholamine molecule; these models predict the same dependence of concentration ratio on $\Delta pH$ and $\Delta\Psi$ , and are therefore thermodynamically equivalent, differing only in the ionic form of the amine that is transported. It seems very likely that uncharged catecholamine/$H^+$ exchange occurs, from experiments measuring the pH dependence of the Michaelis constant ($K_m$) for catecholamines, and using inhibitors of transport such as unprotonated dihydrotetrabenazine (Sherman & Henry, 1983). The transport of catecholamines depends on $\Delta pH$ and $\Delta\Psi$ generated by the $H^+$-ATPase and therefore, at equilibrium :

$$\log ([CAH^+]_{in} / [CAH^+]_{out}) = 2\Delta pH + \Delta\Psi / Z \qquad (2)$$

where Z = 2.3RT/F. Given a $\Delta pH$ of 1.7 (i.e. cytosolic pH = 7.2, matrix pH = 5.5) and $\Delta\Psi$ of +60mV, eqn. (2) predicts a transmembrane catecholamine activity gradient of greater than $10^4$. Amine uptake is essentially irreversible as the carrier is kinetically "gated" by the low intragranular pH, as shown by studies on intact granules and resealed membrane "ghosts" (Maron *et al.,* 1983). Very little efflux of accumulated amine occurs; therefore, the catecholamine activity gradient *in vivo* may be maintained with relatively little energy expenditure.

For studies of catecholamine transport, the exchanger accepts several amines as substrates, and these include adrenaline, noradrenaline and 5-hydroxytryptamine (serotonin), as well as the natural substrate dopamine. Experimentally measured $K_m$ value for amines are generally in the range 5-20μM (Phillips,1974b; Knoth *et al.,* 1981; Carty *et al.,* 1985); however, as the transported catecholamine (unprotonated form) is only a minor species at physiological pH, the true $K_m$ will be very much lower (about 0.1μM) (Sherman & Henry, 1981). Some progress has been made towards the identification of polypeptides involved in catecholamine transport. Covalent labelling of proteins using inhibitors such as 2-[$^3$H]-dihydro-tetrabenazine, and photoreactive affinity labelling using an azido derivative of 5-hydroxytryptamine, has revealed possible candidates of apparent molecular weight 70 and 45kDa, respectively (Sherman *et al.,* 1983; Gabizon & Schuldiner, 1985).

### *1.2.9 Redox reactions in chromaffin granules*

Chromaffin granule membranes contain two major proteins, dopamine ß-hydroxylase (DßH) and cytochrome $b_{561}$ (together accounting for about 40% of the total membrane protein) which are involved in redox reactions. Dopamine ß-hydroxylase is a glycoprotein consisting of four subunits each of molecular weight about 75kDa and having two copper binding sites per subunit (Klinman *et al.,* 1984). The enzyme is a component of both the matrix and the membrane (about 50% activity in each) and catalyses the synthesis of noradrenaline within the matrix as follows:

$$\text{dopamine} + \text{ascorbate} + O_2 \longrightarrow \text{noradrenaline} + \text{semidehydroascorbate} + H_20 \qquad (3)$$

Ascorbate, present at a concentration of 22mM within the matrix, is used as reductant and transfers electrons to the copper ions of DßH to generate the free radical semidehydroascorbate (Skotland *et al.,* 1980; Diliberto *et al.,* 1980).

The semidehydroascorbate undergoes extremely rapid non-enzymic dismutation:

$$2 \text{ semidehydroascorbate} \longrightarrow \text{ascorbate} + \text{dehydroascorbate} \qquad (4)$$

The dehydroascorbate content of the matrix is only about 1% of that of ascorbate content, and it must be reconverted to ascorbate in order for further ß-hydroxylation of dopamine to occur. Reduction of intragranular dehydroascorbate is presumed to occur *in vivo* when electrons flow from the cytosol to the matrix mediated by the enzyme cytochrome $b_{561}$, a very hydrophobic transmembrane protein which is exposed mostly on the cytoplasmic face of the membrane (Duong and Fleming, 1984). Apps *et al.,* (1984) showed that cytochrome $b_{561}$ displays a sigmoidal rather than a linear titration curve, with a midpoint potential of 140mV. It is most likely that the functional enzyme has two or more subunits which interact; the molecular weight of the individual subunits is 30kDa, each subunit consisting of 273 amino acid residues (Perin *et al.*, 1988).

The *in vivo* activity of cytochrome $b_{561}$ has been inferred from "reverse" experiments using ascorbate-loaded granule "ghosts" (Harnadek *et al.,* 1985; Njus *et al.*, 1987b). These authors monitored electron transport, mediated by the enzyme, from internal ascorbate to external ferricytochrome C, and thence to oxygen. The resulting membrane potentials (inside positive) were able to drive reserpine-sensitive noradrenaline uptake. Recently, Kelley & Njus (1988) have analysed the kinetics of electron transfer in this preparation. They obtained rate constants for the oxidation of cytochrome $b_{561}$ by external ferricyanide, and for the reduction of the cytochrome by internal ascorbate. From their values it is clear that the rate of semidehydroascorbate regeneration is much faster than the rate of its disproportionation and therefore, *in vivo*, cytochrome $b_{561}$ mediates electron transfer for the continuous regeneration of ascorbic acid. However, the source of the

extragranular electrons required for the activity of cytochrome $b_{561}$ is still not known, although it may prove to be mitochondrial NADH (Njus *et al.,* 1983). The most likely scheme is that the $H^+$-ATPase-generated membrane potential will favour electron transport into the matrix and the acidic matrix pH will make the redox potential of ascorbate more positive (see Njus *et al.,* 1983, for a discussion of this scheme).

### *1.2.10 Nucleotide Transport*

Although ATP is present at high concentrations within the chromaffin granule matrix (about 130mM, see Table 1.2), relatively little is known about the bioenergetics or significance of its accumulation. Intact granules have been reported to contain an ATP transporter that, like the ATP/ADP exchanger of mitochondria, is inhibited by atractyloside. The chromaffin granule carrier is, however, thought to mediate net uptake of ATP, driven by the $H^+$-ATPase generated membrane potential (Aberer *et al.,* 1978). Moreover, fairly high rates of non-inhibitable transport, probably transmembrane diffusion, and a broad carrier specificity (sulphate, phosphate and phosphoenolpyruvate can also be accumulated), make interpretations of the results difficult (Weber *et al.,* 1983). In contrast, uptake of ATP by chromaffin granule "ghosts" seems to occur by a non-saturable diffusion process and no significant ATP concentration gradient can be achieved (Grüninger *et al.,* 1983). In the cytosol and matrix, ATP and other nucleotides exist in several different protonation and chelation states, and their accumulation can be calculated to be very responsive to even small changes in membrane potential (see Phillips 1987, for a discussion). Grüninger *et al.,* (1983) were unable to show any co-transport of ATP with divalent cations such as $Mg^{2+}$ or $Ca^{2+}$ although, as in intact granules, transport of sulphate, phosphate and phosphoenolpyruvate could be demonstrated. The mechanism of nucleotide accumulation thus remains extremely uncertain.

### *1.2.11 Calcium Transport*

It has been known for more than twenty years that the chromaffin granule matrix contains a significant amount of stored calcium (60-120 nmol.mg$^{-1}$ protein), equivalent to a total intragranular concentration of 20-30mM if all the calcium were free in solution (Borowitz *et al.,* 1965; Borowitz, 1967; Serck-Hanssen & Christiansen, 1973; Phillips *et al.,* 1977). This accounts for at least 60% of the calcium found in the whole adrenal medulla in view of the large numbers of chromaffin granules in this tissue. The high intragranular concentration has been postulated to stabilise catecholamine-ATP complexes (Pletscher *et al.*, 1974) although NMR studies of catecholamines and ATP in the aqueous phases of chromaffin granules do not support this idea (Sen *et al.*, 1979). In 1973, Serck-Hanssen & Christiansen showed that the calcium content of bovine adrenal medulla perfused *in vitro* , increased by about 30% in response to extensive acetylcholine stimulation and that the calcium content of chromaffin granules in particular was doubled. These authors concluded that the granules may remove a significant proportion of calcium from the cytosol and be important in calcium homeostasis in the chromaffin cell. However, the mechanism of calcium accumulation by intact granules and the maintainance of calcium homeostasis is not yet resolved.

Kostron *et al.*, (1977) were the first to demonstrate calcium uptake by isolated granules. These workers incubated partly-purified bovine chromaffin granules with $Ca^{2+}$ (plus a trace amount of $^{45}Ca^{2+}$) and then subjected them to sucrose density gradient centrifugation in order to characterise the different subcellular fractions (mitochondria, microsomes and chromaffin granules) that had accumulated the $^{45}Ca^{2+}$. The uptake into granules was temperature-dependent and saturable: negligible uptake occurred at 0°C and uptake rates saturated at external calcium concentrations above 200μM. The maximal rate of uptake was about 1.0 nmol.min$^{-1}$mg$^{-1}$ protein at 37°C, and uptake was not influenced by $Mg^{2+}$ or ATP.

Kostron *et al.*, (1977) concluded that an energy-independent carrier-mediated process existed that was probably exchanging $^{45}Ca^{2+}$ in the external medium with $Ca^{2+}$ stored within the matrix; they were unable to investigate the kinetics of uptake any further.

Experiments with resealed membrane vesicles ("ghosts") that are devoid of intragranular constituents and endogenous ion gradients have provided more definitive information regarding calcium transport. Phillips (1981) measured a $Ca^{2+}/Ca^{2+}$ exchange activity across the granule membrane with an apparent $K_m$ of 38μM and $V_{max}$ of 28nmol.min$^{-1}$mg$^{-1}$ at 37°C; this exchange was inhibited non-competitively by $Na^+$ and ruthenium red, and competitively by $Mg^{2+}$. ATP did not stimulate the uptake; in fact, it was reported to be slightly inhibitory. No attempt was made in this early work to buffer the free $Ca^{2+}$ in the low micromolar range despite the unphysiologically high $K_m$ found. $Ca^{2+}/Na^+$ exchange catalysed by the same carrier was also demonstrated and found to be unaffected by the generation of an $H^+$-ATPase-dependent membrane potential. It was concluded, therefore, that the exchange was electroneutral, i.e. with a stoichiometry of $Ca^{2+}/2Na^+$. Very similar results were found using both granules purified on iso-osmotic Percoll gradients, and "ghosts" by Krieger-Brauer & Gratzl (1982, 1983). These workers showed that sodium-loaded "ghosts" accumulated $Ca^{2+}$ with an apparent $K_m$ of 0.28μM and $V_{max}$ of 14.5nmol.min$^{-1}$mg$^{-1}$ although they were unable to demonstrate ruthenium red sensitivity of either $Ca^{2+}/Ca^{2+}$ or $Ca^{2+}/Na^+$ exchange.

In order to prove that these membrane $Ca^{2+}$ transport systems are involved in $Ca^{2+}$ homeostasis in the chromaffin cell, as suggested by the work of Serck-Hanssen & Christiansen (1973), one needs to understand the bioenergetics of the process more fully. Krieger-Brauer & Gratzl (1982), using a value for the internal volume of chromaffin granules of 2μl.mg$^{-1}$, estimated the concentration of intragranular $Na^+$ to be 47mM. For an electroneutral exchange, at equilibrium:

$$[Ca^{2+}]_{in} / [Ca^{2+}]_{out} = \left\{[Na^+]_{in} / [Na^+]_{out}\right\}^2 \qquad (5)$$

The cytosolic (extragranular) $Na^+$ concentration is presumably in the low millimolar range (1-5mM); therefore, a $Na^+$ activity gradient (matrix over cytosol) of about tenfold would support a $Ca^{2+}$ activity gradient (matrix over cytosol) of about a hundred-fold. The free $Ca^{2+}$ concentration within the matrix has been measured by Bulenda & Gratzl (1985) to be between 4 and 24μM, which is only about 0.05% of the total matrix $Ca^{2+}$; this is about a hundred-fold higher than the cytosolic free $Ca^{2+}$ concentration (0.1μM) and therefore the equilibrium stated above (eqn.5) seems reasonable. Most of the matrix $Ca^{2+}$ is bound to ATP, glycosaminoglycans and negatively charged proteins such as chromogranin A (Winkler & Westhead, 1980; Reiffen & Gratzl, 1986).

What is the mechanism of $Na^+$ accumulation by the granules? Phillips (1981) monitored $^{22}Na^+$ uptake and efflux coupled to $Ca^{2+}$ movements and suggested that an alternative mechanism for $Na^+$ entry existed in addition to $Na^+/Ca^{2+}$ exchange, but this was not investigated further. In contrast, Burger and his colleagues have proposed the existence of two $Ca^{2+}$ transport systems in the chromaffin granule membrane (Häusler *et al.*, 1981; Niedermaier & Burger, 1981; Burger *et al.*, 1984). One of these systems is an ATP-dependent mechanism driven by the electrochemical gradient of protons ($\Delta\tilde{\mu}_{H^+}$) which is abolished after treatment of intact granules with ammonium ions. These workers tried to distinguish between ATP-stimulated $Ca^{2+}$ uptake into chromaffin granules, mitochondria and microsomes. Uptake into mitochondria was 10-50 times higher than that into granules and the granule $Ca^{2+}$ uptake was also inhibited by ruthenium red, rotenone and amobarbital, compounds that are diagnostic for mitochondrial $Ca^{2+}$ transport. They compared $Ca^{2+}$ and noradrenaline uptake into "lighter" (1.6M sucrose gradient fraction) and "denser" (2.0M sucrose gradient fraction) chromaffin granules and found equal noradrenaline uptake into particles of both densities; however, $Ca^{2+}$ uptake was significantly higher in "lighter" granules which were more contaminated by mitochondria (Niedermaier & Burger, 1981). Therefore, it is difficult to exclude the

possible contribution of mitochondrial $Ca^{2+}$ uptake to that assumed to be due to chromaffin granules.

An ATP-independent $Ca^{2+}/Na^{+}$ exchange was also proposed to be present, supposedly manifested following lysis of intact granules or collapse of ΔpH. This $Ca^{2+}/Na^{+}$ exchange was not seen in intact granules (Burger *et al.*, 1984). Burger (1984) has argued that temperature-dependent progressive granule lysis and disruption of the transmembrane pH gradient is the reason why Kostron *et al.*, (1977) and Krieger-Brauer & Gratzl (1982) failed to demonstrate ATP-stimulated uptake of $Ca^{2+}$ into the chromaffin granule matrix.

If $Ca^{2+}$ is taken up by chromaffin granules in an ATP-dependent manner, what would be the mechanism? Njus *et al.*, (1987a) lists three possibilities: **(1)** electrogenic $Ca^{2+}/Na^{+}$ exchange with a stoichiometry of 1:3 or more; $Ca^{2+}$ uptake and $Na^{+}$ efflux would then be driven by the inside positive membrane potential generated by the $H^{+}$-ATPase; **(2)** a $Na^{+}/H^{+}$ exchange activity that could couple the $H^{+}$-ATPase (catalysing inwardly directed $H^{+}$-translocation) to $Ca^{2+}/Na^{+}$ exchange; **(3)** a $Ca^{2+}$-translocating ATPase could be present in the membrane, hydrolysing cytosolic ATP and pumping $Ca^{2+}$ into the matrix.

However, Flatmark *et al.*, (1985) and Yoon & Sharp (1985) have provided evidence against alternative (3). CaATP can serve as a substrate for the $H^{+}$-ATPase, which has a maximal activity of ATP hydrolysis and $H^{+}$ translocation of about 20% of that measured with MgATP; CaATP or free $Ca^{2+}$ inhibit the basal Mg-ATPase because CaATP functions as a competitive substrate for the $H^{+}$ pump with a lower affinity. Moreover, CaATP driven $H^{+}$-pumping activity was inhibited by N-ethylmaleimide (NEM) at concentrations similar to that found when MgATP is used, and sodium orthovanadate was ineffective. Flatmark *et al.*, (1985) concluded that the $Ca^{2+}$-stimulated ATPase activity was entirely attributable to the $H^{+}$-pump.

Yoon & Sharp (1985) used high resolution proton NMR spectroscopy to monitor the internal pH of chromaffin granule "ghosts" lysed and resealed in the presence of EDTA. Uncomplexed EDTA and Ca-EDTA (which is generated following $Ca^{2+}$ influx

into the ghosts) give rise to distinctive methylene peaks in the NMR spectrum; therefore, intravesicular pH changes following ATPase-induced acidification, and deprotonation of EDTA when the Ca-EDTA complex is formed, could be monitored. $Na^+$-loaded ghosts were able to drive $Ca^{2+}$ uptake in the absence of ATP-driven $H^+$-translocation but no $Ca^{2+}$ uptake occurred in the absence of an opposing $Na^+$ gradient. However, Yoon & Sharp (1985) were able to measure net acidification of the "ghost" lumen upon $Ca^{2+}$ entry, indicating a lack of concomitant $H^+$ extrusion. These results are not consistent with the idea that the endogenous proton gradient is the immediate driving force for $Ca^{2+}$ accumulation, but these authors could not exclude indirect coupling to the proton-motive force, possibly involving a transmembrane $Na^+$ gradient.

Another common divalent cation, $Mg^{2+}$, is also present in the chromaffin granule matrix at a total concentration of about 5mM (Phillips *et al.*, 1977). However, its free concentration has not been measured although it is likely to be very low (Johnson & Scarpa, 1976b; Bulenda & Gratzl, 1985). Some evidence of membrane potential-dependent $Mg^{2+}$ uptake across the granule membrane has been presented (Feidler & Daniels, 1984) but the significance of this ion within the matrix is not clear.

It is now known that secretory vesicles from many other types of secretory cells also possess $H^+$-translocating ATPases in their membranes, together with an acidic matrix and high concentrations of biogenic amines, nucleotides, proteins and divalent cations; however, the proportions of these constituents in each type vary markedly (Table 1.3) (Njus *et al.*, 1987a). This is not an exhaustive comparison and in many cases the constituents of granule matrices and their transport mechanisms remain obscure.

*Table 1.3 Comparison of some secretory granule matrices**

| Component | Bovine Chromaffin granule | Torpedo Cholinergic granule | Pig platelet dense granule | Rat insulinoma granule | Rat parotid granule |
|---|---|---|---|---|---|
| **Amines** | 550mM CA | 870mM ACh | 180mM 5HT | variable | _ |
| **Nucleotides** | 130mM | 120mM | 740mM | 12mM | <10mM |
| **Divalent cations** | 20mM $Ca^{2+}$<br>5mM $Mg^{2+}$ | 170mM $Ca^{2+}$<br>_ | <50mM $Ca^{2+}$<br>1.37 mM $Mg^{2+}$ | 120mM $Ca^{2+}$<br>72mM $Mg^{2+}$<br>23mM $Zn^{2+}$ | 8mM $Ca^{2+}$<br>5mM $Mg^{2+}$ |
| **Protein** | 170mg.$ml^{-1}$ | little | little | 380mg.$ml^{-1}$ | 300mg.$ml^{-1}$ |
| **pH** | 5.5 | 5.7 | 5.7 | 5.8 | 6.8 |
| **Diameter** | 280nm | 90nm | 200nm | _ | 1000nm |

CA = catecholamine 5HT = 5-hydroxytryptamine ACh = acetylcholine

* modified from Njus *et al.*, (1987a)

### *1.2.12 Organisation of the Chromaffin Granule Matrix*

It is of course possible that divalent cations are incorporated at an early stage of granule biogenesis. Chromaffin granules begin life as a collection of proteins in the rough endoplasmic reticulum (RER); these proteins possess signal sequences which direct transport into the lumen of the RER. Here, the signal peptides are removed and the shortened proteins are core-glycosylated, after which they pass into the Golgi apparatus where their sugar moieties are modified. The secretory proteins are subsequently processed and packaged into the newly-formed chromaffin granules. During this maturation process, the granules receive their complement of catecholamines, nucleotides and ions by the operation of the transport processes already described.

It has been proposed by Winkler (1977) that calcium may enter granules during the early stages of their biosynthesis and perform an "aggregation" or "condensation" function by complexing with the newly synthesised proteins; this may also be true for the parotid gland (Wallach & Schram, 1971) and zymogen granules of the pancreas (Ceccarelli *et al.*, 1975). Entry of calcium is postulated to precede that of other small molecules (catecholamines and ATP). In this scheme, a calcium/nucleotide "core" would form and might stabilise the final catecholamine/ATP storage complex of the mature granule (Pletscher *et al.*, 1974). Pulse-chase experiments suggest that new chromaffin granules start to accumulate ATP before acquiring catecholamines (shown by subcellular fractionation of rat adrenal medullae) (Slotkin & Kirshner, 1973). However, no evidence is yet available which demonstrates conclusively the precise sequence of events.

NMR studies have thrown some light on the physical organisation of the matrix of mature granules. Thus, the majority of the matrix proteins (chromogranins) have a random coil configuration and are in rapid motion (for example, the rotational correlation time for the $\alpha$-carbons of the amino acids is 2nsec); values for the

rotational times of the carbon atoms of catecholamines and protons from ATP are even lower (Sharp & Richards, 1977; Sen & Sharp, 1980,1981). These studies indicate that all the catecholamine is in free motion in the aqueous phase of the matrix and that high molecular weight storage complexes of catecholamine/ATP are absent, in contrast to the suggestion of Pletscher *et al.*, (1971).

However, considering the high concentrations of matrix constituents (see Table 1.2), it can be calculated that the intragranular osmolarity would be about 800mosM if all the matrix components were free in solution. Since the osmolarity of the cytosol is about 300mosM, and the granules themselves behave as perfect osmometers (Johnson & Scarpa, 1976b), one would expect a lowering of matrix osmolarity in order to prevent granule lysis. It has been shown by Koppell & Westhead (1982), using vapour pressure osmometry, that ATP-catecholamine solutions are highly non-ideal: thus, a solution consisting of 0.5M adrenaline and 0.16M ATP has an osmotic pressure of only 250mosM at 37°C. Addition of monovalent and divalent cations and of anionic proteins then increased the osmolarity in an almost ideal manner. From these observations, it is envisaged that some sort of catecholamine-ATP interaction occurs which is responsible for the lowering of intragranular osmolarity to that of the cytosol, and that binding of these constituents to the anionic chromogranins (present at high concentrations in the matrix) contributes very little to this (Koppell & Westhead, 1982). The overall interaction of catecholamines and ATP, presumably a ring stacking aided by ionic interaction, is quite short-lived, with rapid exchange occurring between the molecules (Winkler & Westhead, 1980).

## 1.3 *Mechanisms of $Ca^{2+}$ Transport across Plasma and Intracellular Membranes*

Several membrane calcium transport systems are present in all types of cells and are integrally related to their physiological functions. Typically, plasma membranes possess a $Ca^{2+}$ pump ($Ca^{2+}$-translocating ATPase), a $Ca^{2+}$ channel, and a $Ca^{2+}/Na^{+}$ exchanger. Intracellular organelles such as mitochondria contain a $Ca^{2+}$ uniporter, utilised exclusively for $Ca^{2+}$ uptake from the cytosol, and a $Ca^{2+}/Na^{+}$ or $Ca^{2+}/H^{+}$ exchanger different from that of the plasma membrane for $Ca^{2+}$ release from the mitochondrial matrix. Endoplasmic and sarcoplasmic reticulum have specific $Ca^{2+}$-ATPases, different from that of the plasma membrane, for $Ca^{2+}$ sequestration.

Because $Ca^{2+}$ is such a ubiquitous cellular messenger it must be regulated very carefully within the cytosol or within intracellular compartments. The various calcium transport systems are suited to this role, as they have different affinities for interaction with $Ca^{2+}$, and have different total $Ca^{2+}$ transporting capacities. This is a very large subject area, and the reader is referred to the recent review by Carafoli (1987) for more comprehensive information.

### 1.3.1 *The Plasma Membrane $Ca^{2+}$ Channel*

Calcium channels are known to exist in muscle, neuronal and secretory cells. $Ca^{2+}$ influx via voltage-dependent $Ca^{2+}$ channels and $Ca^{2+}$ release from internal stores via $Ca^{2+}$ efflux channels contribute to the rise in the cytoplasmic free $Ca^{2+}$ concentration following stimulation. Based on electrophysiological evidence, it is now clear that there are at least three different types, which have been called L-, N- and T- channels, each one with different properties (Tsien *et al.*, 1988). L-type channels appear to be very abundant, present in most cells and particularly in heart, smooth muscle and skeletal muscle; they are potently inhibited by 1,4-

dihydropyridines, whereas the N- and T- channels are not affected by these compounds.

$Ca^{2+}$ channels are voltage-dependent because they are "gated" by the electrical potential across the plasma membrane. Following depolarisation of the membrane, $Ca^{2+}$ influx occurs maximally at about OmV, allowing the passage of about 3 $x10^6$ $Ca^{2+}$ ions per second (Reuter *et al.*, 1982). 1,4-dihydropyridines, the most frequently used $Ca^{2+}$ channel blockers, are thought to act at the inner surface or within the membrane itself by binding to a specific receptor. The dihydropyridine receptor has been purified from detergent-solubilised transverse tubular membranes of rabbit skeletal muscle, using affinity chromatography. It appears that the skeletal muscle $Ca^{2+}$ channel is composed of at least four subunits, called $\alpha_1$, $\alpha_2$, $\beta$ and $\gamma$ with molecular weights of 175, 170, 52 and 32 kDa, respectively; smaller 24-27 kDa $\delta$-subunits are formed by hydrolysis of the $\alpha_2$-subunit (Takahashi *et al.*, 1987). Regulation of the $Ca^{2+}$ channel by phosphorylation in single heart cells has been studied using the patch clamp technique. Cyclic AMP (cAMP) or the catalytic subunit of cAMP-dependent protein kinase enhance the channel opening probability, leading to an influx of $Ca^{2+}$ (Reuter *et al.*, 1982; Osterreider *et al.*, 1982).

### *1.3.2 The Plasma Membrane $Ca^{2+}/Na^+$ Exchanger*

All cells, with the exception of erythrocytes, contain a $Ca^{2+}/Na^+$ exchanger in their plasma membranes, and this is particularly active in excitable cells such as those from heart, and nervous tissue. The $Ca^{2+}/Na^+$ exchanger is electrogenic, exchanging $3Na^+$ per $Ca^{2+}$; the inwardly directed "downhill" movement of $Na^+$ provides the energy for the outwardly directed "uphill" movement of $Ca^{2+}$. This stoichiometry has been proved conclusively in sarcolemmal vesicles from the heart (Reeves, 1985), in which $Na^+$-loaded vesicles are diluted into a $Na^+$-free medium containing $^{45}Ca^{2+}$, generating an outwardly directed transmembrane $Na^+$ gradient

that drives $Ca^{2+}$ uptake; maximal uptake of $Ca^{2+}$ can be very large, values up to 30 nmoles.$mg^{-1}$ protein.$sec^{-1}$ having been reported (Caroni *et al.*, 1980).

For the case of the sarcolemmal $Ca^{2+}/Na^{+}$ exchange, the cytosolic free $Ca^{2+}$ concentration can be calculated as follows (Reuter, 1985):

$$[Ca^{2+}]_i = [Ca^{2+}]_0 \frac{[Na^+]^3_i}{[Na^+]^3_o} \exp \frac{(n-2)\,FV_m}{RT}$$

where $V_m$ is the transmembrane electrical potential (mV) F, R and T have the usual values, and n is the charge of the transported ion. Under physiological conditions, $[Na^+]_i$ = 6mM, $[Na^+]_o$ = 140mM, $[Ca^{2+}]_o$ = 1mM, $V_m$ -80mV and at 37°C, then $[Ca^{2+}]_i$ would be about 70nM. Because of the electrogenicity of the carrier, $Ca^{2+}$ influx will occur when the membrane is depolarised, with $Ca^{2+}$ efflux occurring as the membrane repolarises.

In the sarcolemmal vesicle preparation, the $K_m$ for $Ca^{2+}$ during $Ca^{2+}/Na^{+}$ exchange has been measured by several groups, and is generally in the range 10-40μM (Reeves & Sutko, 1979; Philipson & Nishimoto, 1980; Caroni & Carafoli, 1983). These values are much higher than would be expected given the level of free $Ca^{2+}$ found in the cytosol of cardiac cells (0.1-0.2μM), and may reflect differences in the properties of the exchanger in the isolated vesicles compared with those prevailing *in vivo* (Reeves, 1985).

### *1.3.3 The Plasma Membrane $Ca^{2+}$-translocating ATPase*

The $Ca^{2+}$ pump of plasma membranes, which is utilised for extruding $Ca^{2+}$ from the cell, is one of the P-type ATPases exhibiting sensitivity to vanadate and possessing a phosphoenzyme intermediate during the catalytic process (Rudnick, 1986; Pedersen & Carafoli, 1987). The $Ca^{2+}$-ATPase of several membrane systems has been purified to homogeneity using calmodulin affinity chromatography (Niggli

*et al.*, 1979; Caroni & Carafoli 1981; Hakim *et al.*, 1982). In erythrocytes the purified enzyme is a single polypeptide of molecular weight 138 kDa (Niggli *et al.*, 1981); it will successfully transport $Ca^{2+}$ when reconstituted into liposomes. The enzyme is activated by calmodulin, although this does not appear to be due to a phosphorylation reaction; rather, a direct interaction occurs between calmodulin and the pump protein.

Physiologically, the $Ca^{2+}$-ATPase is a high affinity, low capacity transport system with a $K_m$ for $Ca^{2+}$ below 1μM, and transport rates of the order of 0.5 nmoles.$mg^{-1}$ protein per sec (Caroni & Carafoli, 1981). In particularly excitable cells such as those from heart, the $Ca^{2+}/Na^{+}$ exchanger may operate in parallel with the $Ca^{2+}$ pump, catalysing bulk $Ca^{2+}$ removal from the cytoplasm. Calmodulin appears to activate the enzyme by binding to a domain that can be released as a result of controlled proteolysis using trypsin (Zurini *et al.*, 1984). As with the $Ca^{2+}$ channel, phosphorylation of the isolated ATPase by cAMP-dependent protein kinase (Neyses *et al.*, 1985) activates the enzyme.

### *1.3.4 Sarcoplasmic and Endoplasmic Reticulum $Ca^{2+}$ Transport*

Both these membrane systems are endowed with P-type $Ca^{2+}$-translocating ATPases (Pederson & Carafoli, 1987) which hydrolyse cytosolic ATP and pump $Ca^{2+}$ into the cisternae, with a probable stoichiometry of $2Ca^{2+}$ translocated per ATP hydrolysed. Most studies have focused upon the sarcoplasmic reticulum (SR) enzyme which can account for up to 90% of the SR membrane protein in some skeletal muscles. In essence, the $Ca^{2+}$-ATPase removes $Ca^{2+}$ from muscle cytosol into the cisternae in resting muscle which leads to the dissociation of $Ca^{2+}$ from Troponin C. Calsequestrin, a 45 kDa ptotein within the lumen of the SR, has the capacity to bind large amounts of $Ca^{2+}$ (about 40 mol/mol) with low affinity ($K_D$ 0.8mM) (MacLennan & Wong, 1971). When muscle is stimulated large amounts of $Ca^{2+}$ are released, triggering muscle contraction through the concerted action of troponin C

and tropomyosin. The ATPase has a high affinity for $Ca^{2+}$ ($K_m$ 0.1-1.0μM) and during the reaction cycle a specific aspartate residue is transiently phosphorylated by ATP. The gene for the $Ca^{2+}$-ATPase from skeletal muscle and heart SR has recently been cloned and sequenced (MacLennan *et al.*, 1985). The enzyme has about ten membrane spanning regions; much of it is on the cytosolic side of the membrane, where the ATP-binding region, the specific aspartate residue that is phosphorylated, and the $Ca^{2+}$ binding domain, are located.

Endoplasmic reticulum (ER) $Ca^{2+}$-ATPase from non-muscle cells also has a high affinity for $Ca^{2+}$ ($K_m$ 1μM), although the precise details of its molecular mechanism are not as well understood as for the SR enzyme. However, ER is a major store of intracellular calcium and $Ca^{2+}$ uptake appears to be regulated by calmodulin and cAMP-dependent protein kinases (Moore & Kraus-Friedmann, 1983; Falmulski & Carafoli, 1984). The release of $Ca^{2+}$ stored in the ER by $IP_3$ is rapid (100-500 msec), and although this is too slow to be of importance for heart and skeletal muscle contraction, it is the probable mechanism of $Ca^{2+}$ release for non-excitable cells such as smooth muscle (Somlyo *et al.*, 1985). $Ca^{2+}$ release is a transient event, due to rapid hydrolysis of $IP_3$ or its phosphorylation to inositol (1,3,4,5) tetrakisphosphate ($IP_4$). In the past 2-3 years, additional metabolic complexity within the phosphoinositide signalling system has been revealed. $IP_3$ may be metabolised by two routes: dephosphorylation by a 5-phosphatase to $IP_2$, with free inositol being eventually produced by further dephosphorylation, or phosphorylation by a 3-kinase to $IP_4$. This latter step is likely to have considerable significance in cellular $Ca^{2+}$ homeostasis, and the possible function of $IP_4$ has generated much speculation and controversy. In brain, $IP_4$ is rapidly synthesised from $IP_3$ (Irvine *et al.*, 1986) although its $Ca^{2+}$ mobilising ability is very weak. Evidence of synergism of action of the two inositol phosphates, however, is growing. $IP_4$ can activate sea urchin eggs, a process that also requires extracellular $Ca^{2+}$ and $IP_3$ (Irvine & Moor, 1986). The suggestion has been made that re-loading of a depleted $IP_3$-sensitive $Ca^{2+}$ pool is dependent on an $IP_4$-regulated mechanism, possibly

involving $Ca^{2+}$ entry across the plasma membrane or redistribution of intracellular $Ca^{2+}$ from other non-$IP_3$ sensitive pools. In rat cerebral cortical slices, muscarinic receptor stimulation leads to a large accumulation of $IP_4$ (Batty & Nahorski, 1987) and this can be augmented by conditions that increase intracellular $Ca^{2+}$ (Baird & Nahorski, 1986).

In addition to the ER, a class of organelle that may also be stimulated to release $Ca^{2+}$ by $IP_3$, has been described. These organelles have been termed "calciosomes", and appear to contain a calsequestrin-like protein, similar to that found in muscle SR (Volpe *et al.*, 1988). Calciosomes, so far observed only in non-muscle cells, appear adjacent to ER as shown from immunofluorescence studies, and are endowed with a $Ca^{2+}$-ATPase recognised by antibodies against the corresponding muscle SR enzyme. As discussed by Irvine (1989) the role of $IP_4$ may be to link the two organelles (the calciosome and the ER) together to regulate intracellular $Ca^{2+}$ mobilisation . However, further studies are obviously required for purification of the calsequestrin-like protein, and to demonstrate that the calciosome is indeed a distinct cytoplasmic organelle involved in intracellular $Ca^{2+}$ metabolism.

### *1.3.5 $Ca^{2+}$ Transport Systems of Mitochondria*

In mammalian mitochondria, three principal mechanisms of $Ca^{2+}$ transport have been described. The route of $Ca^{2+}$ uptake is an electrogenic uniport, $Ca^{2+}$ entering the mitochondrial matrix in response to the membrane potential (inside negative) generated by the activity of the respiratory chain. This $Ca^{2+}$ uniporter is inhibited by very low concentrations of ruthenium red. In the absence of an inorganic anion, such as phosphate, in the external medium, isolated mitochondria take up only limited amounts of $Ca^{2+}$; with phosphate present, "matrix loading" occurs as the rate and extent of $Ca^{2+}$ accumulation is stimulated. The result is the precipitation of insoluble Ca-phosphate deposits, probably hydroxyapatite, in the matrix, which are visible as electron-dense cores in electron micrographs (Greenawalt *et al.*, 1964).

The $Ca^{2+}$ uniport is essentially an irreversible $Ca^{2+}$ uptake mechanism since the transmembrane potential that drives it is unlikely to collapse to values that would stimulate $Ca^{2+}$ efflux. However, the low affinity for $Ca^{2+}$ ($K_m$ 10μM) of the uniporter, and its inhibition by physiological concentrations of $Mg^{2+}$, means that this uptake system will function at well below its maximal capacity given the free concentrations of $Ca^{2+}$ that exist in the cytosol (0.1-0.2μM). It is generally believed that under pathological conditions, such as reperfusion following ischaemia in the heart, excess $Ca^{2+}$ penetrates into the cytosol, with the low-affinity mitochondrial $Ca^{2+}$ uniporter being responsible for the subsequently large total accumulation of $Ca^{2+}$; thus, this system behaves as an essential "safety device", storing excess calcium as an insoluble deposit with phosphate and then releasing it into the cytosol for export from the cell once the injuring condition has subsided (Carafoli *et al.*, 1983).

In many mitochondria, $Ca^{2+}$ efflux occurs by $Ca^{2+}/Na^{+}$ exchange, a process that is independent of the uptake route and not is inhibited by ruthenium red. It is electroneutral, exchanging $2Na^{+}$ for $1Ca^{2+}$, giving a sigmoidal relationship between the external $Na^{+}$ concentration and the rate of $Ca^{2+}$ efflux: half-maximal activity occurs at 4-5mM $Na^{+}$ (Crompton *et al.*, 1976) with a $K_m$ for internal $Ca^{2+}$ between 3 and 6μM (Coll *et al.*, 1982; Hansford & Castro, 1981). Excitable tissues, such as heart and brain, have very active exchangers (Crompton *et al.*, 1976, 1977; Nicholls, 1978) but the exchanger is less active in non-excitable cells such as liver (Nedergaard & Cannon, 1980; Goldstone *et al.*, 1983). The $Ca^{2+}/Na^{+}$ exchanger has been hypothesised to operate asymmetrically in energised mitochondria, with a higher affinity for $Ca^{2+}$ at the matrix than the external side, and/or higher $Na^{+}$ affinity at the external than the matrix face (Crompton, 1985). This was suggested as a result of competitive inhibition of $Ca^{2+}$ binding by $Na^{+}$ at the external face, which decreases the affinity for binding $Ca^{2+}$ quite considerably (Hayat & Crompton, 1982); an analogous situation within the matrix would therefore limit the $Ca^{2+}$ efflux catalysed by the carrier if the exchanger operated symmetrically.

The third route of $Ca^{2+}$ transport is $Ca^{2+}$ efflux that occurs in the absence of external $Na^+$, even when the uptake route is blocked using ruthenium red. This $Na^+$-independent efflux in which $Ca^{2+}$ is exchanged for $H^+$ is prominent in tissues where $Ca^{2+}/Na^+$ exchange activity is lowest, for example in liver and kidney (Akerman, 1978; Nicholls & Crompton, 1980). Liver mitochondria have been shown to catalyse electroneutral $Ca^{2+}/2H^+$ antiport (Brand, 1985). Unfortunately, however, no specific inhibitors of this exchange are known and it is not clear whether a specific $Ca^{2+}/H^+$ antiporter exists.

Regardless of the precise details about the mechanisms and stoichiometry of uptake and efflux, it is now clear from recent electron probe microanalysis studies (Somlyo *et al.*, 1985, 1986), that, *in situ* , mitochondria contain only 1-2 nmoles $Ca^{2+}$ per mg of protein, and that the major role of intramitochondrial $Ca^{2+}$ is to regulate the activity of three matrix dehydrogenases important for oxidative metabolism: pyruvate dehydrogenase, NAD-dependent isocitrate dehydrogenase and $\alpha$-oxoglutarate dehydrogenase (Denton & McCormack 1980; McCormack & Denton, 1986). These enzymes are controlled by oscillations of matrix $Ca^{2+}$ in the micromolar range, and their activity is integrally related to the "$Ca^{2+}$ cycle" in which uptake is driven by the membrane potential (uniport) and efflux occurs in exchange for external $Na^+$ (or $H^+$). Isolated coupled heart mitochondria, for example, suspended in a medium containing physiological concentrations of $Na^+$ and $Mg^{2+}$, maintain their matrix dehydrogenases half- maximally activated at 0.1-0.5$\mu$M external $Ca^{2+}$. The "$Ca^{2+}$ cycle" is envisaged as regulating the matrix enzymes over the same range of free $Ca^{2+}$ as is found in the cytosol of intact tisue (Crompton, 1985).

The $Ca^{2+}$ transport properties that have been described in this section have different kinetic properties, poised to satisfy the different requirements of cells during their functional cycle. Although many cell functions are mediated by oscillations of free $Ca^{2+}$ within the cytosol, these are usually only transient in nature. The time scales of these oscillations do vary, however, ranging from 200-

400 msec in muscle cells which contain high $Ca^{2+}$-affinity sarcoplasmic reticulum, to several seconds in the case of mitochondria, when faced with abnormally high levels of free $Ca^{2+}$ that have penetrated the cell during cell injury.

In general, $Ca^{2+}$ fluxes in cells may be classified in two ways depending on the needs of the cell and their particular functions: high affinity, low capacity systems (exemplified by ATPases) which can regulate cytosol or organelle $Ca^{2+}$ very rapidly and precisely; and low affinity, high capacity systems, which allow slower movements of bulk $Ca^{2+}$. Channels, exchangers and electrophoretic uniporters are all examples of low affinity systems.

### *1.3.6 The $Ca^{2+}$ Transport System of Chromaffin Granules*

To date, the chromaffin granule membrane $Ca^{2+}$ transport system (Phillips, 1981) appears to be similar to that of heart mitochondria (Crompton *et al.*, 1977) catalysing $Ca^{2+}/Ca^{2+}$ as well as electroneutral $Ca^{2+}/Na^{+}$ exchange, with the same carrier catalysing both modes of transport. In these organelles $Ca^{2+}/Ca^{2+}$ exchange displays a high non-physiological $K_m$ (38μM and 13μM, respectively), although this exchange is unlikely to have any role in calcium homeostasis *in vivo*. In addition, Phillips (1981) using chromaffin granule "ghosts", was unable to demonstrate sigmoidicity in the rate of $Ca^{2+}$ efflux induced by $Na^{+}$, which would be expected for an electroneutral ($2Na^{+}/Ca^{2+}$) exchange. However, the $K_m$ (5mM) and $V_{max}$ (26 $nmol.min^{-1}mg^{-1}$ protein at 25°C) for $Na^{+}$ during $Na^{+}$-induced $Ca^{2+}$ efflux was very similar to that observed in heart mitochondria (Crompton *et al.*, 1976). Evidence has already been presented that the chromaffin granule does not possess a $Ca^{2+}$-translocating ATPase such as those found in plasma membranes or sarcoplasmic recticulum, nor does it appear to have a direct ATP-stimulated uptake of $Ca^{2+}$ as described for synaptic vesicles of rat brain (Rahaminoff & Abramovitz, 1978), or for cholinergic synaptic vesicles from *Torpedo* (Michaelson *et al.*, 1980).

Hormone-containing secretory vesicles from bovine neurohypophyses also possess $Ca^{2+}/Ca^{2+}$ and ATP-independent $Ca^{2+}/Na^{+}$ exchange activities (Saemark *et al.*, 1983a,b). The apparent $K_m$ for $Ca^{2+}$ of 0.7$\mu$M during $Ca^{2+}/Na^{+}$ exchange suggests that this transport system may be important for $Ca^{2+}$ removal from the cytosol following stimulation of the nerve terminal.

Storage organelles from a wide variety of secretory cells are known to contain high concentrations of divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ (see Table 1.3, page 37). However, knowledge of their intravesicular function and membrane transport is far from complete, and a detailed understanding of these phenomena is needed in order to appreciate their role in cellular bioenergetics and homeostasis.

*Chapter Two*

# *Materials and Methods*

## *2.1 Materials*

All chemicals were of analytical grade or the highest chemical purity available and were supplied by the Sigma and BDH Chemical Companies, both of Poole, Dorset, UK. All radiochemicals were obtained from Amersham International, Amersham, Bucks, UK. Percoll was from Pharmacia Fine Chemicals, Uppsala, Sweden. Cellulose nitrate filters (pore size 0.45μM) were from Schleicher and Schüll, Dassel, West Germany. High molecular weight polyvinyl chloride (PVC), tetrahydrofuran and "calcium cocktail" containing the $Ca^{2+}$ ionophore 1001 for construction of $Ca^{2+}$-sensitive electrodes were obtained from Fluka Chemicals Ltd., Glossop, Derbyshire, UK. Ionophore A23187 was from Eli Lilley, Indianapolis (USA) and octaethyleneglycoldodecyl ether ($C_{12}E_8$) was obtained from the Kouyoh Chemical Co., Tokyo, Japan.

9-amino-6-chloro-2-methoxyacridine (ACMA) was a gift from Dr. R. Kraayenhof (Vrije University, Amsterdam, The Netherlands) and ethylisopropylamiloride (EIPA) was kindly supplied by Prof. F. Lang, Institut Für Physiologie, University of Innsbruck, Austria. Bafilomycin $A_1$ was generously donated by Dr. K. Altendorf, University of Osnabrück, Osnabrück, West Germany. The computer program used to calculate free $Ca^{2+}$ concentrations was obtained from Dr. A. P. Dawson, School of Biological Sciences, University of East Anglia, Norwich, U.K.

"Buffered sucrose solution" consists of 0.3M sucrose buffered with 10mM Hepes at the pH given. The pH of the Hepes stock solution (1M) was adjusted using NaOH (4M), KOH (4M), tetramethylammonium hydroxide (TMA hydroxide, 2M) or Tris base (2M), as appropriate.

Scintillation fluid used was generally "Optiphase Safe" obtained from LKB, Loughborough, Leicestershire, UK. Alternatively, scintillation fluid containing 5g of 2,4-diphenyloxazole and 0.3g of 1,4-bis-(5-phenyloxazol-2-yl) benzene per litre of toluene was used.

## *2.2 Subcellular Fractionation of the Adrenal Medulla*

### *2.2.1 Crude Granules*

"Crude" chromaffin granules were prepared by differential centrifugation of homogenates of adrenal medulla (Phillips, 1974a). Fifteen to twenty-five bovine adrenal glands were obtained from a local slaughter house within 1 hour of death of the animal and placed on ice. They were transported to the laboratory and chromaffin granules were prepared within the next 2 hours. The inner pink medullae were scraped off the outer brown cortex into 200-300 ml ice cold buffered sucrose: 0.3M sucrose containing 10mM Hepes, pH 7.0 (see Materials). Medullae were passed through a steel mincer with holes of 2mm diameter, diluted using buffered sucrose and then homogenised in a glass homogeniser with a loose fitting teflon pestle at 600 r.p.m. All subsequent procedures were performed at 0-4°C . Cell debris (nuclei, red blood cells, broken membranes, cortical cells, etc) was removed by centrifugation at 4000 r.p.m. ($g_{av}$ = 1,500) for 5 min in a Beckman JA14 rotor. The pellet was discarded and the supernatant centrifuged at 14,000 r.p.m. ($g_{av}$ = 19,000) for 30 min using the same rotor; the supernatant was discarded and at this stage the pellet consists of a lower pink layer of chromaffin granules with an overlaying "fluffy" brown mitochondrial layer. After removal of the latter by washing, granules were resuspended in buffered sucrose, homogenised again, and centrifuged at 15,000 r.p.m. ($g_{av}$ = 18,000) for 20 min in a Beckman JA20 rotor. The washing, resuspension and centrifugation procedure was generally repeated once more to give a "visibly" clean pink granule pellet. This was kept on ice in approximately 5 ml buffered sucrose until used, and constitutes the "crude granule" fraction.

For granules used for matrix $Na^+$ and $K^+$ and $Ca^{2+}$ determinations, the 15,000 r.p.m. step was performed three times with vigorous removal of overlying

mitochondria, by swirling with buffered sucrose before each wash. This process decreases granule yield but markedly increases purity.

### *2.2.2 Purification of Intact Chromaffin Granules on Iso-osmotic Percoll Gradients*

Crude granules (0.5 ml; 10-20 $mg.ml^{-1}$) were layered on top of discontinuous Percoll gradients prepared in 15ml Corex tubes; they were composed of 4 ml 60% Percoll (bottom), 3 ml 45% Percoll (middle), and 4 ml 20% Percoll (top), all prepared in 0.35M sucrose, 20mM Hepes-tris, pH 7.0. After centrifugation at 12,000 r.p.m. ($g_{av}$11,300) in the Beckman JA20 rotor for 20 min at 4°C, fractions were pumped from the bottom of the tube. Chromaffin granules were collected from the 60% Percoll region, and the 60%/45% Percoll interface; mitochondria were collected from the 45%/20% Percoll interface. Fractions were kept on ice and used within a few hours.

### *2.2.3 Preparation of Chromaffin Granule "Ghosts"*

"Ghosts" were prepared by a modification of the method described by Apps *et al.,* (1980). Crude granules (5-10ml) were added dropwise to 340ml of 10mM Hepes or Mes buffer on ice, with stirring. The granules lysed immediately in the hypo-osmotic buffer, and after a few minutes, 60ml 2M sucrose was added to restore the osmolarity to 0.3M. The resealed "ghosts" were collected by centrifugation at 23,000 r.p.m. ($g_{av}$ = 42,000) for 30 min in a Beckman Ti45 rotor; "ghosts" formed a pink outer layer with a darker, central disc of contaminating mitochondrial membranes at the bottom of the tube. The latter were discarded as far as possible, and the resealed "ghosts" were resuspended by gentle homogenisation in buffered sucrose to a final volume of about 20ml. A sucrose density gradient was then used to purify them from any remaining mitochondrial contamination. Gradients were

prepared in Beckman cellulose nitrate tubes (14 x 89mm) by underlaying 4.5ml of 0.4M sucrose/10mM Hepes or Mes with 2.5ml of 0.4M sucrose in $^2H_2O$/10mM Hepes or Mes, using a syringe with a long needle attached. 5ml of the "ghost" suspension was carefully overlayed on top of the gradient, followed by centrifugation at 40,000 r.p.m. ($g_{av}$ = 196,000) for 30 min in a Beckman SW41 rotor; the pink band of purified "ghosts" (3 - 6 $mg.ml^{-1}$) was collected from the 0.4M sucrose/0.4M sucrose in $^2H_2O$ interface and was stored in 0.1 or 0.2ml aliquots at -20°C.

Several different types of "ghosts" were prepared. When "ghosts" were "loaded" at pH 6.0, lysis of granules was performed in a solution containing 10mM Mes-KOH, 0.1mM EGTA-KOH (pH 6.0), and solutions used for resuspension and purification were also buffered with 10mM Mes (pH 6.0), as described above.

$Na^+$-loaded "ghosts" were prepared by lysis of granules in 10mM Hepes-KOH, 0.1mM EGTA-KOH (pH 7.0) containing 25mM $Na_2SO_4$; solutions used in purification then also contained 25mM $Na_2SO_4$ and 10mM Hepes-KOH, pH 7.0.

Some granules were lysed in a solution consisting of 10mM EDTA-TMA, 10mM Hepes-TMA, pH 7.0 to prepare EDTA-loaded "ghosts". Subsequent purification was in sucrose solutions buffered with 10mM Hepes-TMA, pH 7.0.

Finally, $K^+$-loaded "ghosts" were also used. Granules were lysed in 25mM $K_2SO_4$, 0.1mM EGTA-KOH and 10mM Hepes-KOH, pH 7.0. "Ghosts" were purified in sucrose solutions buffered with 10mM Hepes-KOH , pH 7.0 containing 25mM $K_2SO_4$.

### *2.2.4 Adrenal Medullary Mitochondria*

The pellet from the 14,000 r.p.m. centrifugation step was resuspended by gentle homogenisation in buffered sucrose and 20ml aliquots were layered over 50ml 1.8M sucrose, 20mM Hepes-NaOH, pH 7.0 and centrifuged at 45,000 r.p.m. ($g_{av}$ 161,000) for 90 min in a Beckman Ti45 rotor. Mitochondria were recovered from the 0.3M/1.8M sucrose interface, lysed in 20mM Hepes-NaOH pH7.0, and centrifuged at 45,000 r.p.m. for 60 min. The mitochondrial pellets were

resuspended by homogenisation in 20mM Hepes-NaOH, pH 7.0 and after layering 20ml aliquots over buffered 1M sucrose, were centrifuged for 60 min at 45,000 r.p.m. in the Beckman 45Ti rotor in order to remove contaminating chromaffin granule membranes. The pellets were resuspended in a small volume of 20mM Hepes-NaOH, pH 7.0, and frozen at -20°C.

### *2.3 Transport Assays*

Transport of 5-hydroxytryptamine, $Ca^{2+}$, $Na^{+}$ and methylamine into intact chromaffin granules or resealed membrane "ghosts" was measured by incubation in a medium containing the radiolabelled substrate; samples were removed at various time intervals and the granules or "ghosts" were separated from the medium by rapid membrane filtration. In most cases, samples of incubation media (0.1 or 0.2ml) were added to a large excess of ice-cold iso-osmotic quench medium to terminate substrate transport, and then filtered through 2.5 x 2.5 $cm^2$ cellulose nitrate filters (0.45μM pore size). Filters were supported either on a multiport filtration manifold, or on a single sintered glass support, and connected via a trap to a vacuum pump. After washing, filters were generally dried under a lamp and transferred to plastic vials to which 4ml scintillation fluid was added. Radioactivity trapped on the filter was counted in a liquid scintillation counter as described below.

#### *2.3.1 5-hydroxytryptamine Uptake*

ATP-dependent uptake of 5-hydroxy[$^{14}$C]tryptamine ([$^{14}$C]5HT) into intact chromaffin granules or resealed membrane "ghosts" was determined by incubating granules or "ghosts" at 37°C (0.1mg protein.$ml^{-1}$) in a medium containing 0.3M sucrose, 10mM Hepes-KOH, pH 7.0, 6mM ATP ($K_2$ salt), 3mM $MgSO_4$, 58μM [$^{14}$C]5HT (1μCi.$ml^{-1}$). Samples (0.1ml) were withdrawn at intervals to 2.5ml to ice cold 0.3M sucrose, 10mM Hepes-KOH, pH 7.0 and filtered as described above;

they were washed with 2 x 2.5ml 0.3M sucrose, 10mM Hepes-KOH, pH 7.0, dried under a lamp, and radioactivity was determined by scintillation counting.

Simultaneous accumulation of 5HT and $Na^+$ was measured by incubating "ghosts" at 30°C in a medium containing 0.3M sucrose, 30mM Hepes-KOH, pH 7.0, 6mM $ATPK_2$, 3mM $MgSO_4$, 20mM KI, 0.1mM $^{22}Na_2SO_4$ ($3\mu Ci.ml^{-1}$), and 50μM-[$^3$H]5HT ($1\mu Ci.ml^{-1}$). Samples (0.1ml) were removed at intervals to 2.5ml ice cold 0.3M sucrose, 30mM Hepes-KOH, pH 7.0 containing 10μM 5HT. $Na_2SO_4$ was added to 5mM before filtering. Filters were washed with 2 x 2.5ml 0.3M sucrose, 10mM Hepes-NaOH, pH 7.0, dried and counted.

### *2.3.2 $Ca^{2+}$ Uptake*

Uptake of $Ca^{2+}$ was monitored by incubating intact chromaffin granules or "ghosts" (0.1-0.2 mg protein.$ml^{-1}$) in media containing: 0.3M sucrose, 10-30mM Hepes, pH 7.0 adjusted with Tris base, KOH or TMA-hydroxide, as appropriate; $^{45}CaCl_2$ (0.5 - 2.0$\mu Ci.ml^{-1}$) and non-radioactive $CaCl_2$ at various concentrations. $Ca^{2+}$ was either added directly from a stock solution in the absence of a $Ca^{2+}$ chelating agent, or was used in combination with ATP, EGTA, HEDTA or NTA to give various free $Ca^{2+}$ concentrations, as described below. At various time intervals, 0.1ml samples were removed and quenched with 2.5ml ice-cold 0.3M sucrose, 10mM Hepes, pH 7.0 (adjusted using Tris base, KOH or TMA-hydroxide) and 1mM EGTA. Filters were washed with 2 x 2.5ml of cold quench medium lacking EGTA and then counted for radioactivity.

For measuring the initial rate of $^{45}Ca^{2+}$ uptake during $Ca^{2+}/Na^+$ exchange, $Na^+$-loaded "ghosts" (3μl) were placed on the side of a plastic tube (4ml, flat base polypropylene, Sarstedt, Leicester, UK) containing 97μl of incubation medium of the following composition: 0.3M sucrose, 10mM Hepes-KOH, pH 7.00 ± 0.02, $^{45}CaCl_2$ ($0.6\mu Ci.ml^{-1}$) and 0.2mM (nominal) EGTA or HEDTA; $CaCl_2$ was included to give free $Ca^{2+}$ concentrations within the range 0.08 to 9.2μM, as determined using a

$Ca^{2+}$-specific electrode (see below). Uptake was initiated by mixing the pre-warmed (37°C) contents on a vortex mixer, and was terminated at various time intervals by rapid addition of 2.5ml of ice cold 0.3M sucrose, 10mM Hepes-KOH, pH 7.0, containing 1mM EGTA. Filters were washed and counted as above.

### *2.3.3 $Na^+$ Uptake*

Accumulation of $^{22}Na^+$ by a pH jump was achieved by diluting low pH "ghosts" (resealed at pH 6.0) 30-40 fold into media at 25°C at more alkaline pH containing 0.3M sucrose, 20mM Hepes-KOH, 0.1mM EGTA-KOH and various concentrations of NaCl in the presence of $^{22}NaCl$ ($4\mu Ci.ml^{-1}$). At various time intervals samples (0.1ml) were withdrawn and added to 2.5ml ice-cold 0.3M sucrose, 20mM Hepes-KOH pH 7.0; $Na_2S0_4$ was added to 5mM before filtering. Washing of filters was with 2 x 2.5ml 0.3M sucrose, 20mM Hepes-NaOH, pH 7.0; they were then counted for radioactivity. Higher concentrations of $Na^+$ added to the quench solution did not further reduce non-specific $^{22}Na^+$ binding to the filters. Similarly, inclusion of 0.1mM amiloride in the quench solution had no effect on values.

For determining the initial rate of $^{22}Na^+$ uptake during a pH jump a drop (8µl) of "ghost" suspension ($pH_{in}$ = 6.0) was placed on the side of a 4ml flat-base polypropylene tube. Uptake was started by mixing rapidly (Vortex mixer) with 200µl of incubation medium, composed of 0.25M sucrose, 20mM Hepes-KOH, pH 7.0, 0.1mM EGTA and $Na^+$ concentrations in the range 0.5 to 50mM, with $4\mu Ci.ml^{-1}$ $^{22}NaCl$. $K_2SO_4$ was also added to each tube to maintain osmolarity. Uptake was quenched by ading 3.5ml ice-cold 0.3M sucrose, 10mM Hepes-NaOH pH 7.0, containing 5mM $Na_2SO_4$. After filtering, filters were washed with 2 x 2.5ml ice-cold quench medium lacking $Na^+$, dried and counted as above.

### *2.3.4 Blanks*

In all experiments reporting accumulation or efflux of radiolabelled substrate, non-specific binding of the label to the filters has been corrected for. This involves quenching and filtering appropriate volumes of incubation media which are devoid of granule or "ghost" protein. Blanks (duplicate or triplicate samples) were usually 5-10% of the value obtained with membrane protein and these values were subtracted from the uptake or efflux data. In all cases, after filtration the filters were washed with a volume of iso-osmotic quench medium to remove radioactive label that was bound either to the filters or to the membranes. It was found that a 2 x 2.5ml wash was optimum, further washes not significantly reducing the non-specific binding. In $^{45}Ca^{2+}$-containing media, addition of ice-cold quench solution containing a large excess of EGTA (1mM) is sufficient to completely chelate external $Ca^{2+}$, terminating transport and removing externally bound $Ca^{2+}$; this was used by Reed & Bygrave (1975) in experiments on $Ca^{2+}$ transport by mitochondria. Because isolated granules and "ghosts" are osmotically sensitive *in vitro* (Johnson & Scarpa, 1976b; Phillips & Allison, 1978) accumulation of radiolabel into the granule or "ghost" lumen could be assessed by subjecting samples to osmotic shock. Samples were removed from the incubation medium and diluted into the appropriate quench solution, which lacked sucrose as osmotic support; this serves to lyse the "ghosts", releasing the accumulated radiolabel. After a few minutes, sucrose was added to 0.3M and the samples were then filtered and washed in the usual way.

Duplicate or triplicate samples (10μl) of incubation media containing membrane protein were placed on dry filters and counted in order to quantitate the specific radioactivity of incubations, and these values were used in the calculation of uptake or efflux data.

### *2.3.5 Scintillation Counting*

Radioactivity trapped on filters was determined by adding 4 ml "Optiphase Safe" scintillant to a plastic vial containing a filter, followed by counting in a Searle Nuclear Chicago Mark III scintillation counter. Samples were counted for 10 min with individual quench correction and disintegrations per minute (d.p.m.) were calculated automatically from the measured counts per minute (c.p.m.). $^{45}Ca$ was counted in the $^{14}C$ channel of the scintillation counter with an efficiency of 90%. For samples containing $^{3}H$ and $^{22}Na^{+}$ a dual-isotope sorting programme with quench correction was used, giving data as $^{3}H$ c.p.m. and $^{22}Na$ c.p.m.

### *2.3.6 Calculation of Internal Concentrations*

For estimating the accumulated concentrations of $Ca^{2+}$, $Na^{+}$ and amines, I have used a value of $3.6\mu l.mg^{-1}$ protein for the internal volume of "ghosts" (Phillips & Allison, 1978) when using media of low ionic strength, but a value of $3\mu l.mg^{-1}$ protein whem media were supplemented with salt solutions. A value for the internal volume of intact chromaffin granules of $4.3\mu l.mg^{-1}$ protein (Pollard *et al.*, 1976; Phillips *et al.*, 1977) has been used.

## *2.4 Measurement of Free $Ca^{2+}$ Concentrations and pH Gradients in Chromaffin Granules*

### *2.4.1 Determination of Free $Ca^{2+}$ Concentrations*

The concentration of free $Ca^{2+}$ in the intra- and extravesicular compartments in the presence of several interacting ligands has been calculated using a computer

program based on the algorithm of Storer & Cornish-Bowden (1976). This program, which runs on a small Apple IIe microcomputer, takes into account the known association constants of chelating agents (including ATP) with $H^+$, $Ca^{2+}$ and $Mg^{2+}$ (Sillen & Martell, 1971). These are listed in Table 2.1.

| Ligand complex | Ligand (L) | | | |
|---|---|---|---|---|
| | EGTA | EDTA | HEDTA | ATP |
| H-L | 9.46 | 10.23 | 9.72 | 6.50 |
| HH-L | 18.31 | 16.39 | 14.97 | 10.55 |
| HHH-L | 20.99 | 19.06 | 17.01 | 14.59 |
| HHHH-L | 22.90 | 21.05 | - | 18.65 |
| Ca-L | 11.00 | 10.59 | 8.14 | 3.60 |
| CaH-L | 14.79 | 13.74 | 11.10 | 8.30 |
| Mg-L | 5.21 | 8.69 | 5.78 | 4.00 |
| MgH-L | 12.83 | 12.51 | 11.15 | 8.50 |

**Table 2.1**. Logarithm of the association constants of EGTA, EDTA, HEDTA and ATP with $H^+$, $Ca^{2+}$ and $Mg^{2+}$ (Sillen & Martell, 1971).

When NTA was used to buffer free $Ca^{2+}$ concentrations in the absence of other chelating agents, Schwarzenbach's $\alpha$-coefficient method (1957; described in Perrin & Dempsey, 1974) was employed. A value for the Ca-NTA apparent stability constant of 5370 $M^{-1}$ at 37°C and pH 7.0, calculated using the $\alpha$-coefficient method, was used.

### *2.4.2 Measurement of Free $Ca^{2+}$ Concentrations Using a $Ca^{2+}$-specific Electrode*

Alternatively, free $Ca^{2+}$ concentrations in EGTA and HEDTA buffered solutions were determined using a $Ca^{2+}$-specific minielectrode. The electrode membrane I have used consists of polyvinyl chloride (PVC) with the neutral $Ca^{2+}$ ionophore ETH 1001 and tetraphenylboron dissolved in it (Simon *et al.*, 1978). Electrodes were

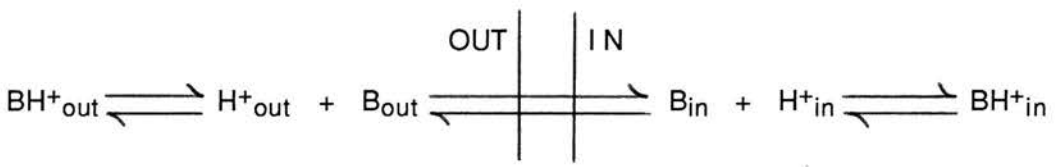
constructed according to the method of Clapper & Lee (1985): pieces of teflon tubing (1.5mm i.d., 3mm o.d., 5cm long) were dipped into a solution consisting of a 10µl aliquot of $Ca^{2+}$-electrode cocktail (containing ETH 1001) and 60µl of 12% (w/v) PVC dissolved in tetrohydrofuran. This viscous "glue" forms a thin membrane at the end of the tubing and was allowed to dry for about 10 min; the electrode was then filled with a $Ca^{2+}$-buffer of the following composition (Tsien & Rink, 1980): 5mM $CaCl_2$, 10mM EGTA, 90mM KCl, 10mM Mops, pH 7.3; the free $Ca^{2+}$ was 0.1µM. The electrode was stored in this buffer (> 12 hrs) until used. A silver wire was inserted into the electrode (without touching the membrane), which was connected to a Radiometer pHM 26 pH meter operating in the millivolt output mode. A standard Radiometer Ag/AgCl reference electrode filled with 3M KCl was also used.

The electrode was calibrated according to the method described by Bers (1982), in which the apparent Ca-EGTA and Ca-HEDTA association constants ($K'_{Ca}$) as well as total [EGTA] and total [HEDTA] are determined, rather than relying on published values of $K'_{Ca}$ obtained under different conditions. Measurements were made by dipping the electrodes into solutions in a small plastic vessel of capacity 1.2ml, and open to the air. The temperature of the solutions was maintained at 37°C and they were stirred continuously using a magnetic stirring flea. First, a set of standard $Ca^{2+}$ solutions (0.1 to 10mM) lacking EGTA or HEDTA was used to determine the slope of the electrode, and then a series of $Ca^{2+}$-EGTA and $Ca^{2+}$-HEDTA buffers were employed across a range of free $Ca^{2+}$ concentrations in order to check the linearity and sensitivity of the electrode. The calibration solutions contained, in a volume of 1.0ml: 0.3M sucrose, 10mM Hepes-KOH, pH 7.00 ± 0.02 (adjusted using KOH), nominal 1.0mM EGTA or 0.2mM HEDTA (acid form), and 0.3 - 2.0mM or 0.03 - 0.15mM $CaCl_2$ for EGTA and HEDTA containing solutions, respectively; the potential recorded by the electrode ($V_{Ca}$, mV) was measured for each solution. The free [$Ca^{2+}$] predicted for these solutions was calculated using the association constants for EGTA and HEDTA given by Sillen & Martell (1971). From these estimated free [$Ca^{2+}$], and the known concentrations of the chelating agents, the bound $Ca^{2+}$ concentration at

each point was calculated. Each value was then plotted in a Scatchard plot (see Fig. 5.1, Chapter 5), which was used to determine $K'_{Ca}$ and the concentration of the chelating agent (n). These values then allowed recalculation of free $[Ca^{2+}]$ from the total $[Ca^{2+}]$, using the α-coefficient method of Schwarzenbach (1957), as described in Perrin & Dempsey (1974).

### *2.4.3 Measurement of pH Gradients Across the Chromaffin Granule Membrane*

In the experiments described in this thesis, weak bases have been employed to monitor ΔpH changes across the chromaffin granule membrane. This is based on the assumption that only the uncharged (non-protonated) form (B) of the base is freely permeant, and therefore present in equal concentrations on both sides of the membrane. If the membrane is impermeant to the charged (protonated) form ($BH^+$), the distribution of the various species will be governed by the Henderson-Hasselbalch equation. Therefore, if one side of the membrane is more acidic than the other, the protonated form $BH^+$ will be trapped on that side (Nicholls, 1982):




I have used the weak bases methylamine and ACMA, which have $pK_a$ values of 10.62 and 8.99, respectively. These $pK_a$'s are sufficiently higher than the internal pH's of the granule or "ghost" lumen; therefore, the total equilibrium concentration of methylamine or ACMA will be much higher inside than in a supporting medium at pH 7.0 and the following relationship may be applied:

$$\Delta pH = \log ([B]_i / [B]_o) \qquad (1)$$

where i and o represent the intravesicular and extravesicular compartments, respectively.

### *2.4.4 Acidification of Chromaffin Granule "Ghosts"*

The acidification of $Na^+$-loaded "ghosts" was measured by the membrane filtration method described by Apps *et al.,* (1980). "Ghosts" were diluted into a $Na^+$-free medium (25°C) containing 0.3M sucrose, 30mM Hepes-TMA, pH 7.3, 0.1mM EGTA-TMA, 50mM choline chloride, and 2.5μM [$^{14}$C]methylamine (0.15μCi.ml$^{-1}$). The final $Na^+$ concentration in the medium following dilution was 1.7mM. Samples (200μl) were withdrawn at various time intervals and filtered directly through 13mm$^2$ cellulose nitrate filters (pore size 0.45μM). Filtration was complete in < 5 sec. The wet filters were transferred without washing or drying to scintillation vials and 4ml scintillant was added. Samples were counted as described previously with individual quench correction.

Methylamine accumulation by "ghosts" following a pH "jump" was measured by diluting low pH "ghosts" ($pH_{in}$ = 6.0) into a medium containing 0.3M sucrose, 30mM Hepes-KOH, pH 8.8, 0.1mM EGTA-KOH, 0.5mM $Na_2SO_4$ and 2.5μM [$^{14}$C]methylamine (0.15μCi.ml$^{-1}$) for 5 min at 25°C. The final pH of the medium was 8.0, as determined on a parallel solution using a pH electrode. Samples (200μl) were removed for filtration and counting, as above.

### *2.4.5 Measurement of the Matrix pH of Intact Granules*

The internal pH of intact granules was determined by incubating freshly isolated granules (0.5 mg.ml$^{-1}$) in 0.3M sucrose, 10mM Hepes-TMA, pH 7.3 and 2.5μM [$^{14}$C]methylamine (0.15μCi.ml$^{-1}$) for 5min at 25°C. After chilling on ice for a few minutes, 200μl samples were filtered directly and counted, as above.

### *2.4.6 Calculation of pH Gradients*

In all experiments, the distribution ratio of methylamine between the inside and outside compartments, and therefore the value of ΔpH, was calculated as follows (Phillips & Apps, 1980).

The methylamine concentration inside the granules or "ghosts" ($[CH_3NH_3^+]_i$, in $nmol.\mu l^{-1}$) is given by:

$$[CH_3NH_3^+]_i = [CH_3NH_2]_{total} \times (d.p.m._{in} / d.p.m._{total}) \times (1/ v_i) \qquad (2)$$

where $[CH_3NH_2]_{total}$ is the methylamine concentration ($nmol.ml^{-1}$) of the incubation medium; $d.p.m._i$ is the radioactivity (d.p.m.) trapped on the filter after filtering a volume of granules or "ghosts", minus the radioactivity trapped after filtering the same volume of an identical mixture lacking granule or "ghost" protein; $d.p.m._{total}$ is the total radioactivity of an equal volume of incubation medium to that filtered; and $v_i$ is the internal volume of the granule of "ghost" lumen in $\mu l.ml^{-1}$ of incubation mixture. This value is derived from the protein concentration of the incubation medium and the granule or "ghost" internal volume, taken as $4.3\mu l.mg^{-1}$ protein for granules (Phillips *et al.*, 1977) and either 3.65 or $3\mu l.mg^{-1}$ protein for "ghosts" (Phillips & Allison, 1978).

The external methylamine concentration at equilibrium ($[CH_3NH_3^+]_o$, in $nmol.\mu l^{-1}$) is given by:

$$[CH_3NH_3^+]_o = \frac{[CH_3NH_2]_{total}}{1000} \times \frac{(d.p.m._{total} - d.p.m._i)}{d..p.m._{total}} \qquad (3)$$

dividing eqn. (2) by eqn. (3) gives:

$$[CH_3NH_3^+]_i \; / \; [CH_3NH_3^+]_o \quad = \quad (d.p.m._{in} \; / \; d.p.m._o) \; X \; (1000 \; / \; v_i) \qquad (4)$$

where $d.p.m._o$ is equal to $(d.p.m._{total} - d.p.m._{in})$

The logarithm of the methylamine concentration ratio ($[CH_3NH_3^+]_i$ / $[CH_3NH_3^+]_o$ ) is equal to the magnitude of the $\Delta pH$ generated. No correction for methylamine binding was made. This may cause some error in the calculated $\Delta pH$. Errors may also arise from inaccuracy in the determination of the internal volume (4.3 $\pm$ 1.1 $\mu l.mg^{-1}$).

### *2.5 Fluorescence Assays*

#### *2.5.1 Fluorescent Experiments Using ACMA*

Schuldiner *et al.,* (1972) demonstrated the use of acridine dye fluorescence quenching for studying the internal pH of chloroplasts, and Salama *et al.,* (1980) used this in a detailed study of the internal pH of chromaffin granules. ACMA is a suitable dye for this type of study. It is a fluorescent weak base that accumulates within the acidic matrix of the granule with quenching of fluorescence, due largely to concentration-dependent dye interaction, but also to interaction with ATP (Salama, *et al.*, 1980).

For isolated chromaffin granules or resealed "ghosts" suspended in a medium that has a higher pH than the matrix pH, ACMA is accumulated in the granules and its fluorescence is quenched. Assuming that the $pK_a$ of ACMA (8.99) is the same inside the granules or "ghosts" as it is in the suspending medium, and is high compared with $pH_o$, and also that the fluorescence is completely quenched within the granules or "ghosts" (and that this is unaffected by $pH_i$), then:

$$\log [Q/(100-Q)] \quad = \quad \Delta pH \; - \; \log \; (v_o \; / \; v_i) \qquad (5)$$

(incomplete fluorescence quenching or binding may affect the accuracy of the method).

where ΔpH is the pH difference between the matrix and the medium (outside minus inside), Q is the percentage quenching of ACMA fluorescence, $v_i$ is the internal volume of the granules of "ghosts" and $v_o$ is the volume of the suspending medium (Schuldiner *et al*, 1972).

All experiments were performed using granules or "ghosts" (0.07-0.1mg.ml$^{-1}$) in a Perkin-Elmer 3000 fluorimeter, with excitation at 420nm (slit width 5nm) and emission at 525nm (slit width 10nm). Fluorescence traces were recorded continuously on a Servoscribe potentiometric chart recorder. Fluorescence quenching as a function of external pH (Warnock *et al.*, 1982) was measured in media (25°C; final volume 2.0ml) containing 0.3M sucrose, 30mM Mes or Hepes, 0.1mM EGTA and 0.75μM ACMA, at various pH values between 6.1 and 8.3,* adjusted using KOH or TMA-hydroxide. The pH was measured immediately after each experiment using a pH electrode. Results were plotted according to eqn. (5), as log [Q/(100-Q)] against ΔpH.

### *2.5.2 Fluorescence Assay of $Na^+/H^+$ Exchange*

For monitoring $Na^+/H^+$ exchange activity, Mes-loaded "ghosts" ($pH_{in}$ = 6.0) were diluted into media (25°C; final volume 0.5ml) containing 0.3M sucrose, 10mM Hepes-KOH, pH 7.0, 0.1mM EGTA-KOH, 0.75μM ACMA and NaCl or LiCl, with choline chloride to make up the salt concentration to 60mM. Accumulation of external $Na^+$ in exchange with internal $Na^+$ was manifested as a recovery of ACMA fluorescence (ie, loss of quenching) following the pH jump.

Acidification of $Na^+$-loaded "ghosts" was monitored by adding "ghosts" to media (25°C; final volume 0.5ml) containing 0.3M sucrose, 30mM Hepes-TMA, pH 7.3, 0.1mM EGTA-TMA, 50mM choline chloride and 0.75μM ACMA. The final $Na^+$ concentration in the medium was 1.7mM, and the quenching of ACMA fluorescence was continuously monitored. At various time points following dilution, the pH gradient that is developed across the "ghost" membrane (outside minus inside) can be

* The proximity of this value to the $pK_a$ of ACMA will introduce an error into the use of equation (1).

determined from the plot of eqn. (5), and compared directly with the pH gradient that is measured using the methylamine distribution technique under the same conditions (see section 2.4.4).

### *2.5.3 Free Concentrations of $Na^+$ $K^+$ and $Ca^{2+}$ in the Chromaffin Granule Matrix Measured Using ACMA*

In the experiments described here, freshly isolated "crude" granules in 0.3M sucrose, 10mM Hepes-TMA, pH 7.0 were used. These are relatively stable and mitochondrial contamination has been reduced by vigorous washing. The approach is based on ionophore-mediated electroneutral fluxes of $H^+$ and the particular cation ($Na^+$, $K^+$ or $Ca^{2+}$) under investigation. ACMA is a suitable probe for this type of study.

#### *2.5.3.1 Free $K^+$*

The ionophore nigericin equilibrates $K^+$ and $H^+$ across the membrane; protons enter or leave the granules in the presence of nigericin depending on the concentration gradient of $K^+$ across the membrane, and such fluxes can be followed using ACMA.

In the case of measurements on $K^+$, the media contained, in a volume of 2.0ml: 0.3M sucrose, 10mM Mes-TMA, pH 6.5, 0.1mM EGTA-TMA 0.75μM ACMA, 1mM $Na_2SO_4$ and $K_2SO_4$ in the range of 0.5 to 4.0mM (i.e., 1.0 - 8.0mM $K^+$). On addition of granules (0.1mg.$ml^{-1}$) there is an immediate quenching of ACMA fluorescence (see Fig. 6.2, Chapter 6), the magnitude of which is governed by the size of the pH gradient (eqn. 5). After 3 minutes nigericin was added to 25nM, which results in a movement of $K^+$ ions and a compensatory flux of protons as equilibrium is established. This is reflected by an increase or decrease of ACMA quenching, depending on the value of $[K^+]_o$; $Q^*$ is the new percentage of quenching at

equilibrium.

At this point:

$$[K^+]_i / [K^+]_o = [H^+]_i / [H^+]_o = [ACMA]_i / [ACMA]_o \qquad (6)$$

and, by analogy with eqn. (5),

$$\log[Q^*/(100-Q^*)] = -\log[K^+]_o + \log[K^+]_i - \log (v_o/v_i) \qquad (7)$$

Readings were taken for a further 3 minutes after ionophore addition, steady traces being obtained after 0.5 - 1 minute. The volume ratio is a constant. For small variations in $[K^+]_o$ there will be a small variation in $[K^+]_i$, but this will be approximately proportional to $[K^+]_o$ so that a plot of log $[Q^*/(100-Q^*)]$ against log $[K^+]_o$ should be a straight line.

The null point is defined as that value of $[K^+]_o$ that produces no change in ACMA quenching on addition of the ionophore. At this point $Q = Q^*$ and $pH_i$ is unchanged. Therefore, knowing $pH_i$, $pH_o$ and $[K^+]_o$, $[K^+]_i$ can be calculated.

Eqn. 7 can be generalised for any ionophore that is specific for protons and for a single cation $M^{x+}$:

$$\log[Q^*/(100-Q^*)] = -1/x\ (\log[M^{x+}]_o - \log\ [M^{x+}]_i) - \log\ (v_o/v_i) \qquad (8)$$

Whether a plot of log $[Q^*/(100-Q^*)]$ against log $[M^{x+}]$ gives a straight line depends on how far the internal concentration of the ion is buffered.

### *2.5.3.2 Free $Na^+$*

The same procedure was used for determining the null point for $Na^+$, using the electroneutral $Na^+/H^+$ ionophore monensin, instead of nigericin. Media contained, in a volume of 2ml: 0.3M sucrose, 10mM Hepes-TMA pH 6.5, 0.1mM EGTA-TMA, 0.75μM ACMA and $Na_2SO_4$ in the range 0.75 - 4.0mM (i.e., $Na^+$ in the range 1.5 - 8.0mM). In addition, media were supplemented with $K_2SO_4$ to give a concentration of $K^+$ equal to the $K^+$ null point determined for that batch of granules. Monensin was used at 1.25μM. Results were plotted as log $[Q^*/(100-Q^*)]$ versus log $[Na^+]_o$; the null point was determined by reading the value of Q from the log $[Q^*/(100-Q^*)]$ axis to give a value of $[Na^+]_o$. As $pH_i$ and $pH_o$ are known, $[Na^+]_i$ can be calculated.

### *2.5.3.3 Free $Ca^{2+}$*

For the $Ca^{2+}$ null point determination, the ionophore A23187 (which catalyses $Ca^{2+}/2H^+$ exchange) was used. Free $Ca^{2+}$ concentrations in the external medium were set using $Ca^{2+}$-EGTA buffers, as determined using the computer program described in Chapter 2. The media (2ml final volume) consisted of 0.3M sucrose, 10mM Hepes-TMA, 5mM EGTA-TMA, 0.75μM ACMA and 0.05 - 0.8mM $CaCl_2$. After addition of $CaCl_2$ the pH was re-adjusted to 6.5 using HCl or TMA-hydroxide. This gives free $Ca^{2+}$ concentrations in the range 20 - 400nM. A23187 was used at 1.25μM; a slight correction was necessary for its fluorescence. Results were plotted as $\log[Q^*/100-Q^*]$ against $\log[Ca^{2+}]_o$. An attempt was also made to determine the null point for $Mg^{2+}$, also using A23187. Free $Mg^{2+}$ concentrations in the range 20 - 120nM were obtained in solutions containing 0.3M sucrose, 10mM Hepes-TMA, 5mM EDTA-TMA, pH 6.5 (adjusted using TMA or HCl) 0.75μM ACMA and 10 - 60μM $MgCl_2$. $CaCl_2$ was also added to give a free concentration of $Ca^{2+}$ equal to the null point previously determined using that batch of granules.

## *2.6 Other Methods*

### *2.6.1 Total Content of $Na^+$ and $K^+$ in Chromaffin Granules*

Fresh granules prepared in 0.3M sucrose buffered with either 10mM Hepes-TMA, Hepes-NaOH or Hepes-KOH, pH 7.0, were used (see section 2.2.1). 1ml of granule suspension (20-35mg.ml$^{-1}$) was centrifuged for 5 min at 4°C in a TLA-100.2 rotor of a Beckman TL-100 ultracentrifuge at 100,000 r.p.m. ($g_{av}$ 356,000). The supernatant was discarded and the pellet resuspended in 0.3-0.5ml 0.5% (w/v) aqueous $C_{12}E_8$ by homogenising in a hand-held homogeniser. Lysed granules were vortex-mixed for 2 min and stored at -20°C. For flame photometric analysis of $Na^+$ and $K^+$, samples were thawed, recentrifuged in the TLA-100.2 rotor and the supernatant carefully removed. In some cases, the lysis procedure was repeated and the second supernatant was also recovered for analysis. No significant quantities of $Na^+$ or $K^+$ were detected, however. $Na^+$ and $K^+$ in the supernatant were analysed in an IL543 (Instrumentation Laboratory (UK) Ltd., Altrincham, Cheshire, U.K.) flame photometer with 3M-$LiNO_3$ as an internal standard. Each sample was analysed in triplicate.

For calculating the total matrix concentrations of $Na^+$ and $K^+$, a value for the exchangeable water space inside the granules of 4.3µl.mg$^{-1}$ protein has been used (Pollard *et al.*, 1976; Phillips *et al.*, 1977).

### *2.6.2 Protein Estimation by Dye Binding*

All protein determinations were performed using the method of Bradford (1976). A 5X stock solution of Bradford reagent was made by dissolving 25mg of Coomassie Brilliant Blue (Serva Blue G) in 25ml of 96% ethanol. 50ml of 85% orthophosphoric acid was added and the solution stirred for 60 min at room

temperature. The volume was adjusted to 100ml with distilled water and then filtered through glass wool. Stock solutions were stored at 4°C. A standard calibration plot was constructed over the range 1-10μg bovine serum albumin in a volume of 0.1ml. To each sample was added 1ml 1X Bradford reagent. Protein concentrations were calculated from readings of the absorbance of dye at 595nm taken after 10 min (and before 60 min).

### *2.6.3 Cytochrome Oxidase Assay*

Cytochrome *c* oxidase (EC 1.9.3.1), as a marker for mitochondria, was assayed by following the continuous oxidation of reduced cytochrome c, using a modification of the method described by Mason *et al.*, (1973). Cytochrome c (10mg of Sigma Type III horse heart) was dissolved in 1ml of 50mM Mes-NaOH, pH 6.5, 0.1M KCl, 1mM EDTA-NaOH, that had previously been depleted of $O_2$ by degassing and bubbling with $N_2$. After reducing with a few crystals of sodium dithionite, the sample was desalted through a small (2cm x 1cm) column of Bio-Gel P6DG prepared in a microfuge tube with a hole in the bottom. The Bio-Gel was equilibrated with the above buffer (without cytochrome c). The reduced cytochrome was diluted to 30ml with $N_2$ saturated assay buffer that contained 50mM Mes-NaOH pH 6.5, 1mM EDTA-NaOH and 0.5% (w/v) Tween 80.

Oxidation of reduced cytochrome c by cytochrome oxidase was monitored in a Pye Unicam SP1800 spectrophotometer at 550 nm and compared to an identical reference sample that additionally contained 1mM KCN in Tris-$SO_4$, pH 7.4. Cytochrome c oxidase activity, expressed in $nmoles.min^{-1}.mg^{-1}$ protein, was calculated using a molar extinction coefficient for cytochrome c of 29,500* $mol^{-1}$ l $.cm^{-1}$ (van Gelder and Slater, 1962).

*This number was used by mistake and the difference extinction coefficient is applicable here with a value of 20,000 $mol^{-1}.l.cm^{-1}$. Absolute values for cytochrome oxidase activity should therefore be corrected by a factor of 29.5/20.

### *2.6.4 Acetylcholinesterase Assay*

Acetylcholinesterase (EC 3.1.1.7) was assayed by a modification of the method described by Potter (1967). Assays were performed in plastic scintillation vials, and the assay medium contained, in a volume of 0.1ml: 0.3M sucrose, 100mM Hepes-KOH, pH 8.0, and 40μM [$^3$H]acetylcholine (2.4μCi.ml$^{-1}$). This volume included 20μl of acetylcholinesterase-containing sample. After 10 min at 37°C, the reaction was terminated by the addition of 0.1ml ice-cold stop mix containing 1M-chloroacetic acid, 2M NaCl and 0.5M NaOH. 4ml of a 9:1 (v/v) mixture of toluene fluor and amyl alcohol was added, samples were vortex mixed for 2 min, and then centrifuged at 3000 r.p.m. for 5 min. This extracts [$^3$H]acetate into the organic layer. Whole mixes were then counted in a liquid scintillation counter.

### *2.6.5 Catecholamine Assay*

Catecholamines were measured by a modification of the fluorimetric method of vonEuler and Lishajko (1961) using adrenaline and noradrenaline bitartrate standards (5-50μM). Catecholamines were oxidised by incubating samples (1ml) with 0.75mM $K_3(CN)_6$ in 10mM potassium phosphate buffer, pH 6.2 at room temperature. After 3 min, 2ml of a solution containing 3.5M NaOH, 2% (v/v) ethylenediamine and 0.2% (w/v) ascorbic acid was added and the formation of fluorescent lutines was assayed within 1 hour in a Perkin Elmer 3000 fluorimeter.

For estimating the loss of granular adrenaline (and ATP), granules (0.23mg.ml$^{-1}$) were incubated at 37°C in 0.3M sucrose, 10mM Hepes-tris, pH 7.0 and at various time intervals 0.5ml samples were removed, chilled on ice for a few minutes and then spun at 3000 r.p.m. for 5 min. The supernatants were discarded and the granule pellets resuspended in 0.5ml 10mM Hepes-tris, pH 7.0. The concentrations of adrenaline and ATP were calculated from the absorbance of both

compounds using the formulae:

$$(A_{250} - (A_{291} / 2.4))/ 11.8 = \text{mM ATP} \quad (9)$$

$$A_{291} / 0.82 = \text{mM adrenaline} \quad (10)$$

where 11.8 and 0.82 are the extinction coefficients ($mmoles.l^{-1}.cm^{-1}$) for ATP at 250nm and adrenaline at 291nm, respectively.

### *2.6.6 Sucrose Gradients*

The sucrose gradient described in Chapter 4 was prepared as follows. A linear sucrose density gradient (0.3-1.3M in 30mM Hepes-KOH, pH 7.0) was poured from a gradient mixer with the mixing chamber being magnetically stirred, and the solution being pumped into Beckman cellulose nitrate 13.2ml centrifuge tubes using a peristaltic pump. The upper and lower gradient concentrations for the two mixing chambers were calculated using the formula:

$$C_b = C_a - (V_t / V_g) \times (C_{bottom} - C_{top}) \quad (11)$$

where the concentration in the mixing chamber ($C_a$) equalled the concentration at the bottom of the gradient ($C_{bottom}$), $V_t$ is the total volume of both mixing chambers, $V_g$ is the volume of the gradient (12.8ml), $C_b$ is the concentration in the diluting chamber, and $C_{top}$ is the concentration at the top of the gradient. Gradients were stored at 4°C for 3 hrs before use.

Chromaffin granule "ghost" suspensions (1ml, in 0.3M sucrose, 30mM Hepes-KOH, pH 7.0) that had accumulated radiolabelled substrate were loaded on top of the gradient and centrifuged at 196,000g for 150min at 4°C in an SW41 rotor in a Beckman ultracentrifuge. The tubes were punctured, 0.6ml fractions were

collected, and 0.4ml portions were added to 2.5ml ice-cold 0.3M sucrose, 10mM Hepes-KOH, pH 7.0 for filtration and counting of radioactivity, as described previously. Sucrose densities were calculated from refractive index measurements (using an Abbe 60 refractometer) made on a parallel gradient loaded with buffer only.

### *2.7 Statistical Treatment of Results*

Experiments presented in this thesis are typical from a series performed on several preparations of granules or "ghosts". Data are presented as means ± S.E.M. for the number of investigations (n) carried out, as appropriate. The filtration assays give results reproducible ± 5% (S.E.M. for amines and $Ca^{2+}$), or ± 15% for $^{22}Na^{+}$, in which the ratio of accumulated d.p.m. to background is much lower.

*Chapter Three*

# *$Ca^{2+}$ Transport by Chromaffin Granules*

## *3.1 Introduction*

Chromaffin granules can be purified from homogenates of adrenal medulla. First, a large or "crude" granule fraction is obtained by differential centrifugation which contains, in addition to chromaffin granules, mitochondria, lysosomes and some microsomal elements. Intact granules are best separated from other subcellular organelles by using sucrose density gradients. Continuous sucrose gradients (1.2M to 2.0M sucrose) afford excellent separation from mitochondria, which remain at the top of the gradient, and moderately good separation from lysosomes, which equilibrate at about 1.6M (Smith & Winkler, 1966). Chromaffin granules equilibrate at higher density (about 1.8M sucrose) due to their high protein content. However, the granules behave as osmometers and shrink during the centrifugation procedure as they are relatively impermeable to sucrose (Perlman, 1976). When granules are centrifuged over a layer of 1.6M sucrose a pellet of fairly pure granules is obtained with good yield; purity is improved, although yield is reduced, if 1.8M sucrose is used instead (Bartlett & Smith, 1974).

For studies of membrane and matrix components, the above purification scheme presents few problems, as fairly pure membrane preparations are obtained. However, for studies of membrane transport properties granule integrity must be preserved. This often involves incubation in iso-osmotic media (0.3M sucrose, for example); granules purified on a sucrose density gradient tend to lyse during the process of re-hydration, when transferred to media of lower osmolarity.

Under iso-osmotic conditions using sucrose and metrizamide mixtures, chromaffin granules, lysosomes and mitochondria have rather similar relative buoyant densities (1.12, 1.15 and 1.17, respectively; Morris & Schovanka, (1977)). In such gradients, chromaffin granules of high purity equilibrate above mitochondria although the yield is low and the granule population is relatively non-homogenous. However, most recent work on membrane transport has utilised intact granules purified on iso-osmotic sucrose-Percoll gradients. Percoll consists of polydisperse colloidal silica particles

(mean diameter 17nm) coated with a layer of polyvinyl pyrrolidone (PVP). Terland *et al.*, (1979) achieved a good separation of chromaffin granules, mitochondria and lysosomes using a self-generating Percoll gradient (50% Percoll in 0.5M sucrose) and Meyer & Burger (1979) obtained granules as a pellet following centrifugation through a 20% to 60% Percoll gradient. The contamination with mitochondria and lysosomes was less than for granules isolated over 1.6M sucrose although the yield was lower.

For studies of $Ca^{2+}$ transport by secretory organelles both hyper-osmotic sucrose and iso-osmotic sucrose-Percoll gradients have been used. Burger and his colleagues (Haüsler *et al.*, 1981; Burger *et al.*, 1984) incubated "crude" chromaffin granules with $^{45}Ca^{2+}$ and then purified them on a sucrose density gradient (1.3M to 2.5M sucrose), followed by transfer to 0.3M sucrose before scintillation counting. In addition to the presence of mitochondrial contaminants this procedure renders the granules fragile due to rehydration, as described above; therefore, accumulated $Ca^{2+}$ and other matrix constituents may be lost. In addition, an accurate kinetic analysis of transport is impossible as incubation has to be restricted to a single time point prior to centrifugation.

Using self-generating sucrose-Percoll gradients, Gratzl and co-workers have made a study of $Ca^{2+}$ transport by purified adrenal chromaffin vesicles and pituitary neurohypophyseal vesicles (Krieger-Brauer & Gratzl, 1982; Saermark, *et al.*, 1983a,b). Following the Percoll-gradient step, granules were collected by centrifugation; the resuspended pellets had residual Percoll removed from them by gel filtration. The purified vesicles were then sedimented again and resuspended in iso-osmotic medium for analysis of $^{45}Ca^{2+}$ uptake. These authors demonstrated $Ca^{2+}/Ca^{2+}$ and ATP-independent $Ca^{2+}/Na^{+}$ exchange, but amounts of $Ca^{2+}$ accumulated were rather low and initial rates of uptake could not be measured accurately; this may have been due to the pelletting and resuspension procedure which inevitably damages the vesicles.

The results presented in this chapter describe some aspects of $Ca^{2+}$ transport by bovine chromaffin granules purified on a discontinuous sucrose-Percoll gradient. Granule fractions were collected from the gradient and added directly to incubation media

without removal of Percoll. Notwithstanding some problems of leakage of contents, and therefore of the precise matrix composition, these results are similar both qualitatively and quantitatively, to $Ca^{2+}$ transport processes that have been previously described in chromaffin granule "ghosts" (Phillips *et al.,* 1981; Krieger-Brauer & Gratzl, 1983).

## *3.2 Results*

### *3.2.1 Purification of Intact Chromaffin Granules*

A "crude" granule fraction was centrifuged through a discontinuous gradient of 20, 45 and 60% Percoll in 0.35M sucrose/10mM Hepes-Tris, pH 7.0, and the distribution of particulate material following fractionation of the gradient is shown in Fig. 3.1. Fifteen 0.7ml fractions were collected, and chromaffin granules were recovered from the bottom of the gradient (fractions 1-3 in the 60% Percoll region) and from the 60/45% Percoll interface (fractions 5-7) as indicated by the distribution of adrenaline and noradrenaline. Mitochondria were well-separated from chromaffin granules and were recovered in a region of lower density in the 20% Percoll region (mainly in fractions 10-13) as shown by the distribution of cytochrome *c* oxidase activity. In both chromaffin granule regions the adrenaline content is more than that of noradrenaline and between fractions 8 and 12 the content of both catecholamines declines in parallel. The large amount of adrenaline, noradrenaline, and cytochrome oxidase activity at the top of the gradient is not associated with particulate material and is probably attributable to fragmentation of granules during centrifugation.

During routine fractionation of Percoll gradients, fractions 1-3, 5-7 and 10-13 were pooled to give three fractions that together accounted for most of the "crude" granule protein loaded onto the gradient and about 60-70% of its volume. Percoll was not routinely removed from fractions as identical results were obtained if Percoll was removed in an additional centrifugation step. The total catecholamine content of the interface fraction (2.24 $\pm$ 0.13 $\mu$mol.mg$^{-1}$ protein ; mean $\pm$ S.E.M for three granule preparations, see Table 3.1) is identical to that found by Phillips *et al.,* (1977) for granules purified through 1.6M sucrose. In this fraction 80% of the catecholamine is accounted for by adrenaline. However, noradrenaline is distributed evenly throughout the three regions (i.e. there is no separation of adrenaline and noradrenaline containing granules).

*Fig. 3.1 Percoll gradient analysis of chromaffin granules*

Crude chromaffin granules were subjected to sucrose-Percoll gradient centrifugation as described in Chapter 2. Samples of the fractions collected were analysed for adrenaline (●) and noradrenaline (○) content, or were assayed for their cytochrome *c* oxidase activity (△). Fraction densities (□) were calculated from refractive index measurements made on a parallel gradient.

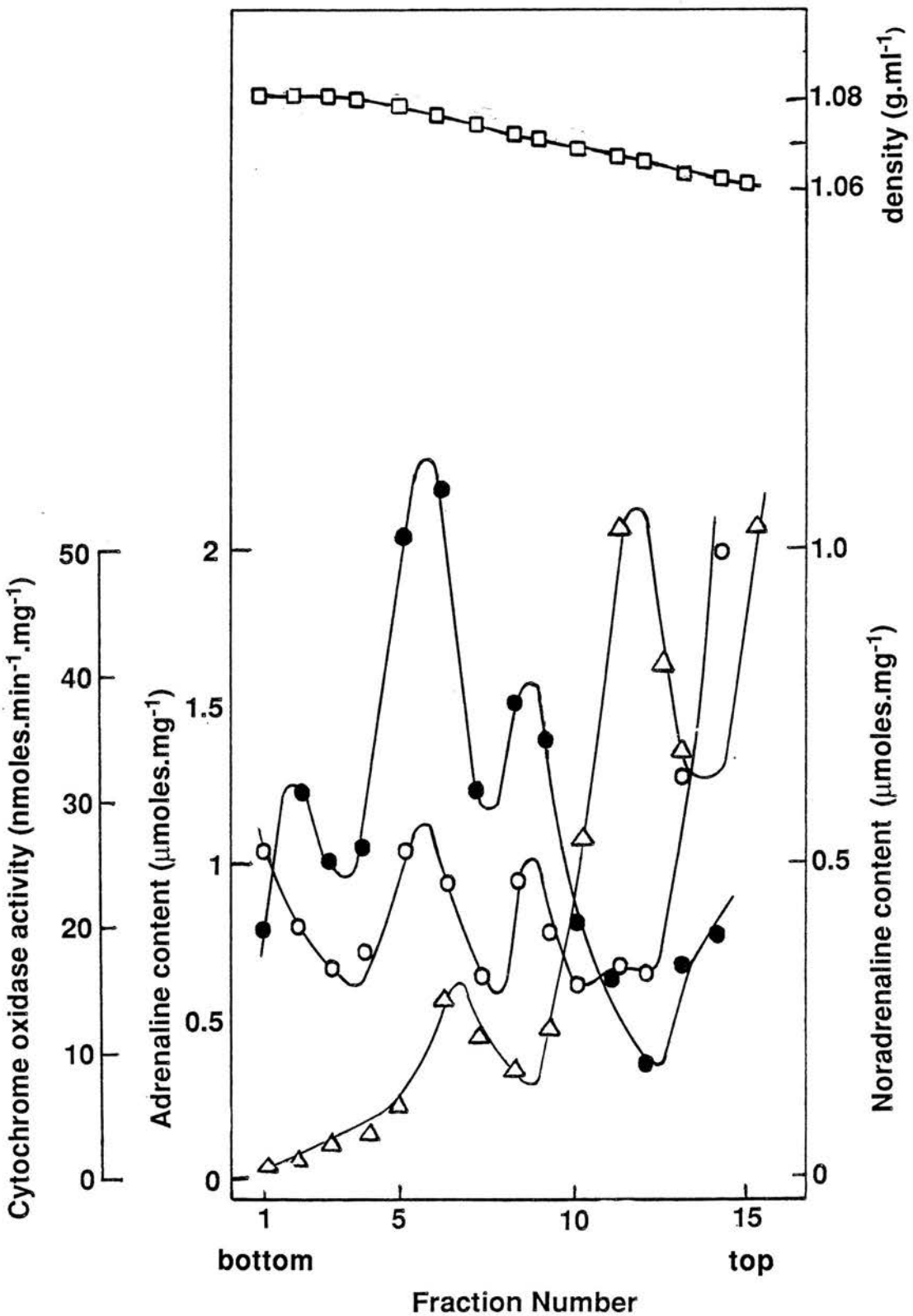

density (g.ml-1)
1.08
1.06
Cytochrome oxidase activity (nmoles.min-1.mg-1)
50
40
30
20
10
0
Adrenaline content (μmoles.mg-1)
2
1.5
1
0.5
0
Noradrenaline content (μmoles.mg-1)
1.0
0.5
0
1
5
10
15
bottom
top
Fraction Number

*Table 3.1 Analysis of subcellular fractions from the adrenal medulla purified on a discontinuous sucrose-Percoll gradient.*

| Fraction | Adrenaline ($\mu$mol.mg$^{-1}$) | Noradrenaline ($\mu$mol.mg$^{-1}$) | Cyt. ox. nmol.min$^{-1}$.mg$^{-1}$ |
|---|---|---|---|
| chromaffin granules (60% Percoll) | 0.98 ± 0.16 (3) | 0.45 ± 0.02 (3) | 2.85 ± 0.47 (13) |
| chromaffin granules (60/45% Percoll) | 1.80 ± 0.11 (3) | 0.43 ± 0.015 (3) | 7.32 ± 1.3 (12) |
| mitochondria (20% Percoll) | 0.64 ± 0.096 (3) | 0.39 ± 0.05 (3) | 20.8 ± 2.43 (12) |
| "crude granules" | – | – | 26.34 ± 2.9 (7) |

Values given are mean ± S.E.M. for the number (n) of different granule preparations. Cyt. ox. = Cytochrome *c* oxidase activity. For details of assays see Chapter 2.

Although markers for chromaffin granules are fairly heterogeneously separated throughout the gradient, good separation of the main bands of chromaffin granules from mitochondria has been achieved. Two bands of granules are obtained because a discontinuous Percoll gradient was used. Contamination of the granule fractions by mitochondria is low. Comparison of cytochrome *c* oxidase activities of "crude" granule and Percoll-purified granules (Table 3.1) with a preparation of purified adrenal medullary mitochondria showed the following percent contamination: "crude granules", 2.7%; 60% Percoll granules, **0.3%**; 60/45% Percoll interface granules, **0.7%**.

For studies of membrane transport, granules recovered from the 60/45% interface region were used. They were kept on ice and used within a few hours.

### *3.2.2 Stability of Chromaffin Granules*

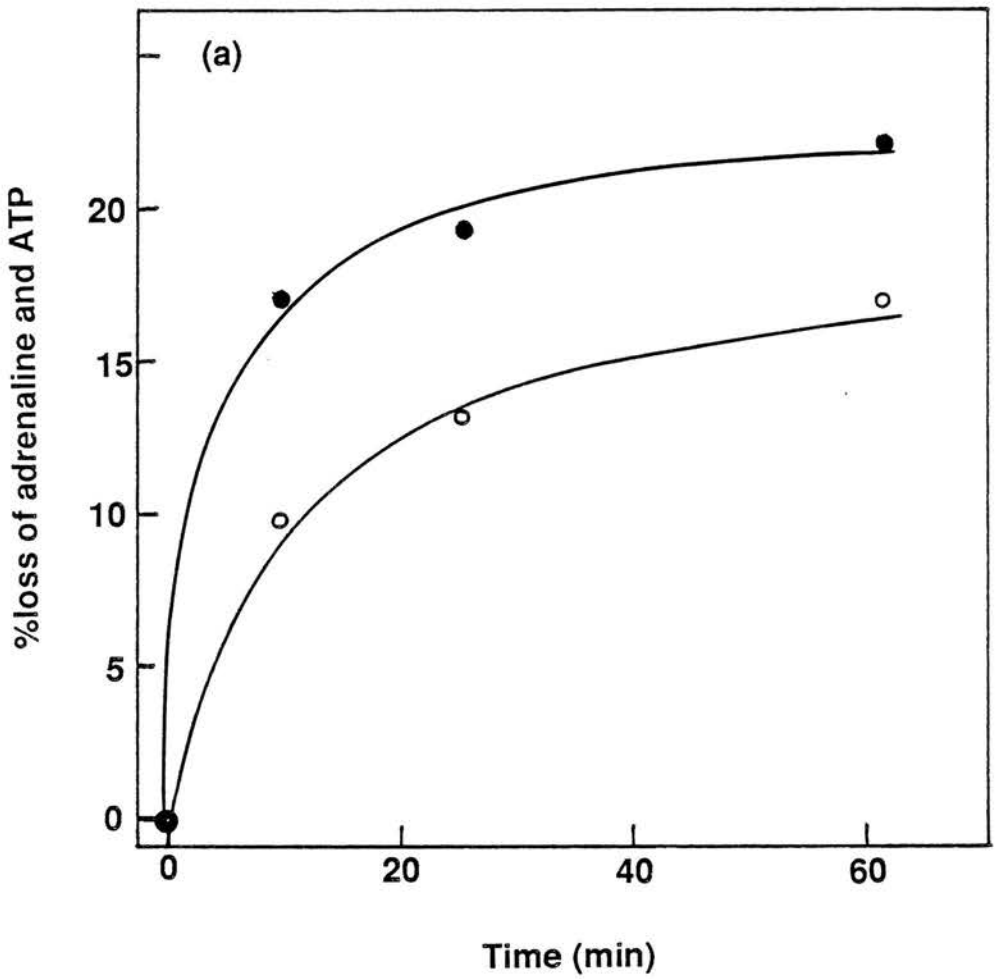
When incubated over a 3h period at 37°C, chromaffin granules gradually lose their matrix constituents. I measured the release of adrenaline and ATP, and after 60 min about 25% of these components has been lost (Fig. 3.2a). However, the sum of the granule pellet and supernatant ATP or adrenaline remained constant throughout the incubation period (results not shown). The rate of leakage is similar to that found by Gratzl *et al.,* (1981), although one cannot tell from Fig. 3.2a whether the loss is due to progressive granule lysis or leakage from intact granules.

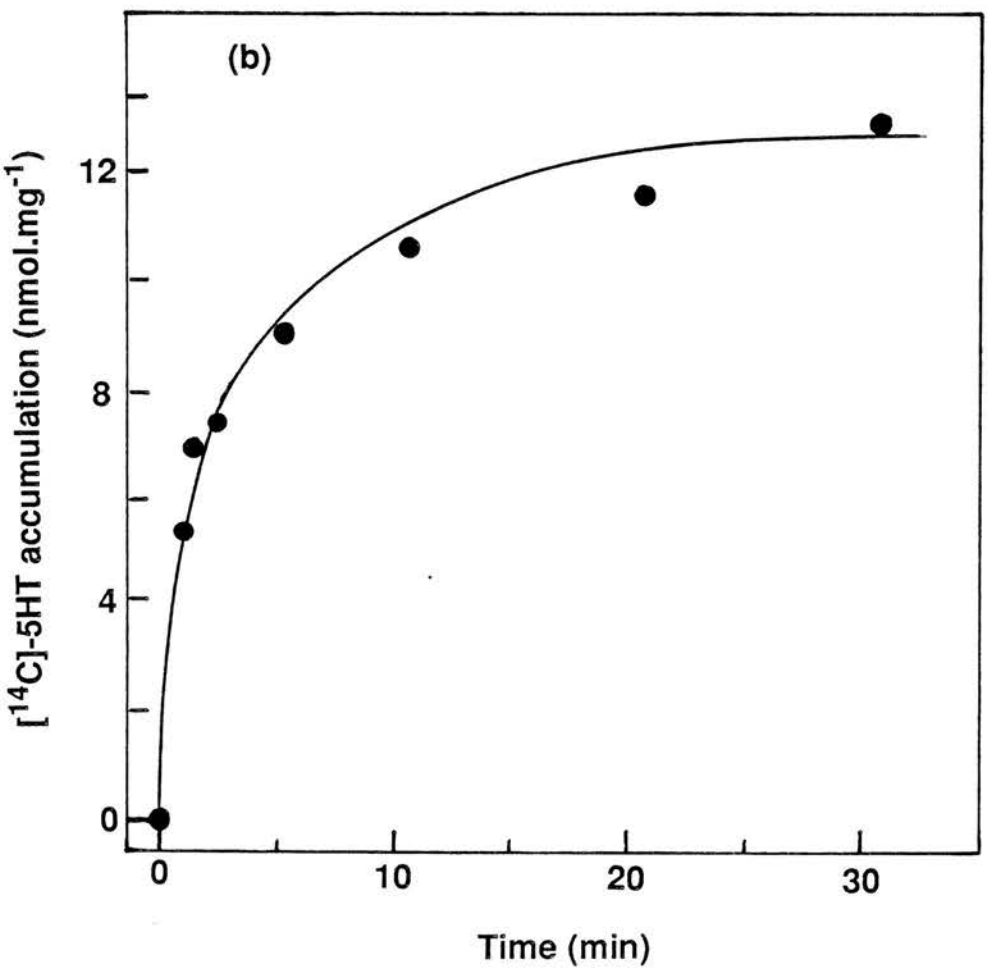
When isolated chromaffin granules, suspended in buffered sucrose solutions at pH 7.0, are incubated with ATP and radiolabelled 5-hydroxytryptamine, a large accumulation of this substrate occurs because of the presence of an acidic matrix pH (about 5.5) and the generation of a transmembrane potential ($\Delta\Psi$, inside positive) of about 80 mV by the membrane's $H^+$-translocating ATPase (Johnson & Scarpa, 1979; Salama *et al.*, 1980). As shown in Fig. 3.2b, when Percoll-purified granules were incubated at 37°C with MgATP and 50μM [$^{14}$C]5HT (in the absence of a permeant anion) a plateau value of about 13nmol.mg$^{-1}$ is reached after about 30 min; this is equivalent to an internal concentration of 3.25mM, and a concentration gradient across the membrane of about 65-fold (inside minus outside) which is maintained for a further 20 min. Thereafter, the content of granular [$^{14}$C]5HT declines, presumably because of granule lysis and dissipation of the proton electrochemical gradient. Consistent with this view is the observation that after 3hrs at 37°C no accumulation of 5HT occurs when granules are incubated with MgATP and 5HT (results not shown).

Slotkin *et al.*, (1971) observed that little or no dopamine ß-hydroxylase (an intragranular enzyme) was released from "crude" granules incubated under similar conditions to those shown in Fig. 3.2a; however, adrenaline was lost at a similar rate. This suggests that the adrenaline (and ATP) release measured here is due to progressive granule lysis rather than leakage from intact granules.

***Fig. 3.2 Stability of chromaffin granules at 37°C***

In **(a)**, progressive loss of adrenaline (●) and ATP (○) from granules (0.23 $mg.ml^{-1}$) was estimated after removing samples from the incubation medium at various time intervals; results are expressed relative to the granule content at the start of the incubation (taken as 100%); In **(b)**, granules (0.26$mg.ml^{-1}$) were incubated in buffered sucrose, pH 7.0 with MgATP and 50μM [$^{14}$C]5HT, and uptake of 5HT was monitored.


(a)
%loss of adrenaline and ATP
0
5
10
15
20
0
20
40
60
Time (min)


(b)
[14C]-5HT accumulation (nmol.mg-1)
0
4
8
12
0
10
20
30
Time (min)

It seems therefore, that the granule preparation used in these studies, although largely devoid of contaminating mitochondria, is probably not as stable as freshly isolated "crude" granules. This renders transport experiments difficult, and most measurements of $Ca^{2+}$ and 5HT transport were completed within the first 60 min of the start of incubations.

### *3.2.3 Calcium Accumulation by Granules*

Incubation of granules in a buffered sucrose medium containing $^{45}Ca^{2+}$ leads to accumulation of $Ca^{2+}$ with an initial rate that is maintained for about 1 min (Fig. 3.3); a steady uptake is observed with a gradual approach to a plateau value after about 30 min. This is followed by a decline in $^{45}Ca^{2+}$ content, and after 3 hours of incubation most of the accumulated $Ca^{2+}$ has been released. The incorporation of $Ca^{2+}$ is assayed by withdrawing samples and adding them to cold buffered sucrose containing EGTA. This serves to chelate $Ca^{2+}$ in the medium and remove it from the external surface of the granule (Reed & Bygrave, 1975). At $0^{o}C$ the membrane has a low ion permeability and therefore accumulated $Ca^{2+}$ is retained within the matrix. Granules are then separated from the medium by rapid filtration through nitrocellulose filters, and $^{45}Ca^{2+}$ is counted. About 75% of incorporated $^{45}Ca^{2+}$ is released from the granules when they are subjected to osmotic shock (by omitting sucrose from the quenching solution).

In six preparations of granules a plateau value of 6.0 $\pm$ 0.22 $nmol.mg^{-1}$ protein (mean $\pm$ S.E.M.) was reached, with 40$\mu$M $CaCl_2$ in the external medium. In fact, an exchange of external $^{45}Ca^{2+}$ for internal $Ca^{2+}$ is occuring across the granule membrane. This is shown (Fig. 3.3) by incubating granules under identical conditions but in the presence of non-radioactive $Ca^{2+}$. After 25 min a trace amount of $^{45}Ca^{2+}$ is added, and uptake is observed at a rate similar to that seen in granules in the original radioactive medium. Since uptake rates are moderately high but total radioactive $Ca^{2+}$ reaches a plateau, there must be an equally high efflux rate. That this radioactive $Ca^{2+}$ is located within the granule matrix is suggested by the osmotic sensitivity of most of the

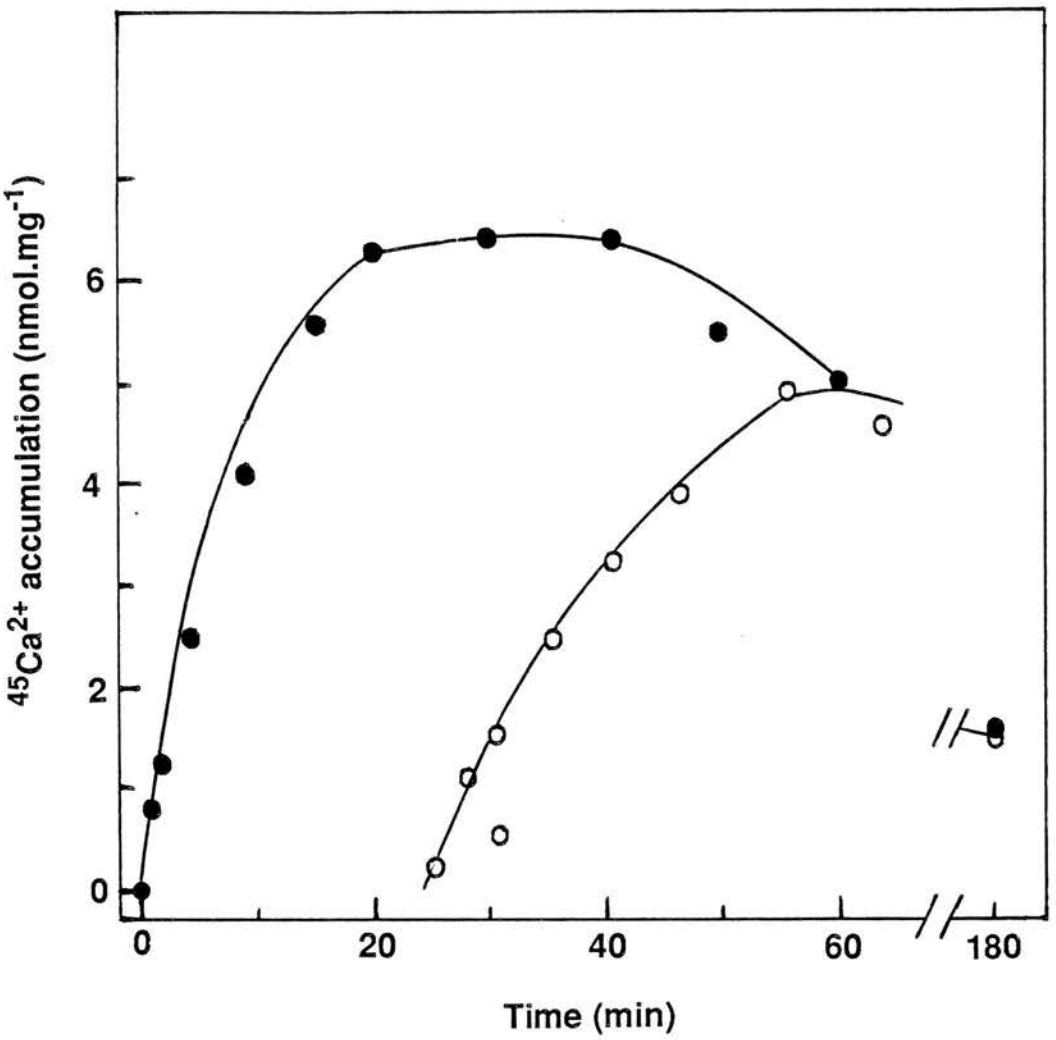



***Fig. 3.3 $Ca^{2+}$/ $Ca^{2+}$ exchange by granules***

Granules (0.26mg.ml$^{-1}$) were incubated at 37°C in a medium containing 40μM (unbuffered) $Ca^{2+}$ plus $^{45}Ca^{2+}$ (0.5μCi.ml$^{-1}$), and samples were withdrawn at intervals for assessment of $Ca^{2+}$ accumulation (●). A parallel incubation was maintained under identical conditions except that $CaCl_2$ was non-radioactive; $^{45}CaCl_2$ (0.4μCi.ml$^{-1}$) was added 25 min (○) after the start of the incubation.

accumulated $Ca^{2+}$, and its insensitivity to EGTA in the quench medium. $Ca^{2+}$ uptake was markedly temperature-sensitive: initial rates of uptake were decreased by about 60% at 20°C, and more than 90% at 0°C.

I measured the initial rates of $^{45}Ca^{2+}$ uptake with various unbuffered external $Ca^{2+}$ concentrations (Fig. 3.4a) and derived an apparent $K_m$ for $Ca^{2+}/Ca^{2+}$ exchange. Uptake was saturable with a $K_m$ for $Ca^{2+}$ of 37 ± 4μM and $V_{max}$ 1.1 ± 0.1 nmol.mg$^{-1}$ min$^{-1}$ (mean ± S.E.M. for three preparations of granules; Fig. 3.4b). This $K_m$ value compares with a value of 38μM obtained with "ghosts" (Phillips, 1981). Sodium ions were found to inhibit $Ca^{2+}$ exchange non-competitively with a $K_i$ of 7.8mM ( $K_i$ 10.7mM in "ghosts") although $K^+$ was not inhibitory (Fig. 3.4b). These results suggest that $Na^+$ may be important in modulating $Ca^{2+}$ transport across the granule membrane. In their experiments with intact granules, Krieger-Brauer & Gratzl (1982) obtained a value of 34mM for the half-maximal inhibition of $Ca^{2+}$ uptake by $Na^+$.

When NTA was used to buffer the external free $Ca^{2+}$ concentration within the range 6-50μM, a $K_m$ for $Ca^{2+}$ during $Ca^{2+}/Ca^{2+}$ exchange of 13μM and $V_{max}$ 1.33 nmol.min$^{-1}$.mg$^{-1}$ was found (Fig. 3.5). Crompton *et al.*, (1977) obtained a very similar value for the $K_m$ for $Ca^{2+}$ at the external face of the mitochondrial $Na^+/Ca^{2+}$ exchanger, when operating in the $Ca^{2+}/Ca^{2+}$ exchange mode.

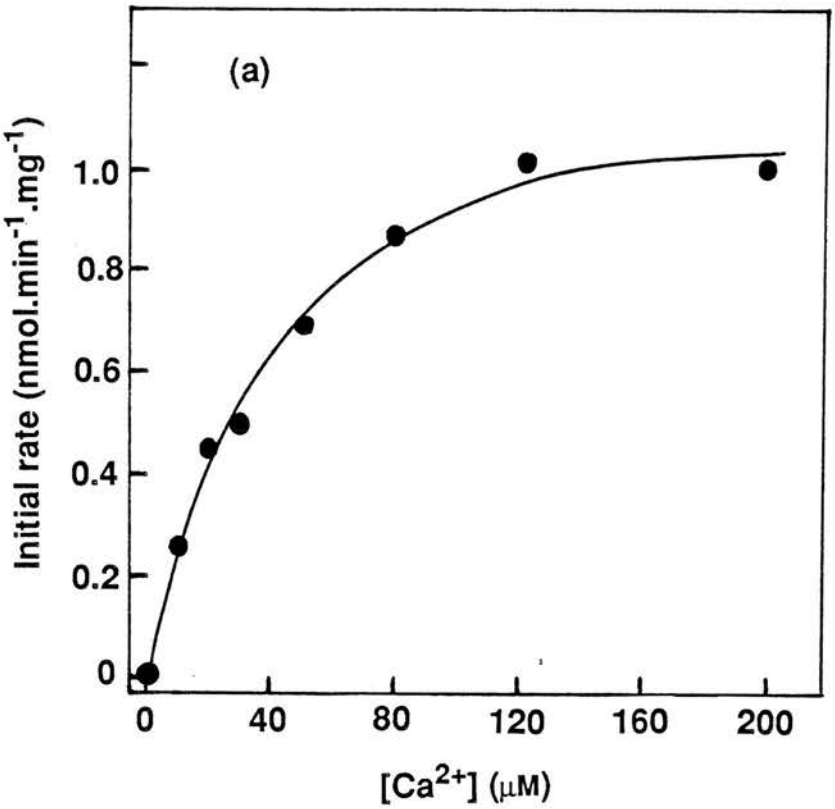
### *3.2.4 $Na^+$- and EGTA-induced Efflux of $Ca^{2+}$*

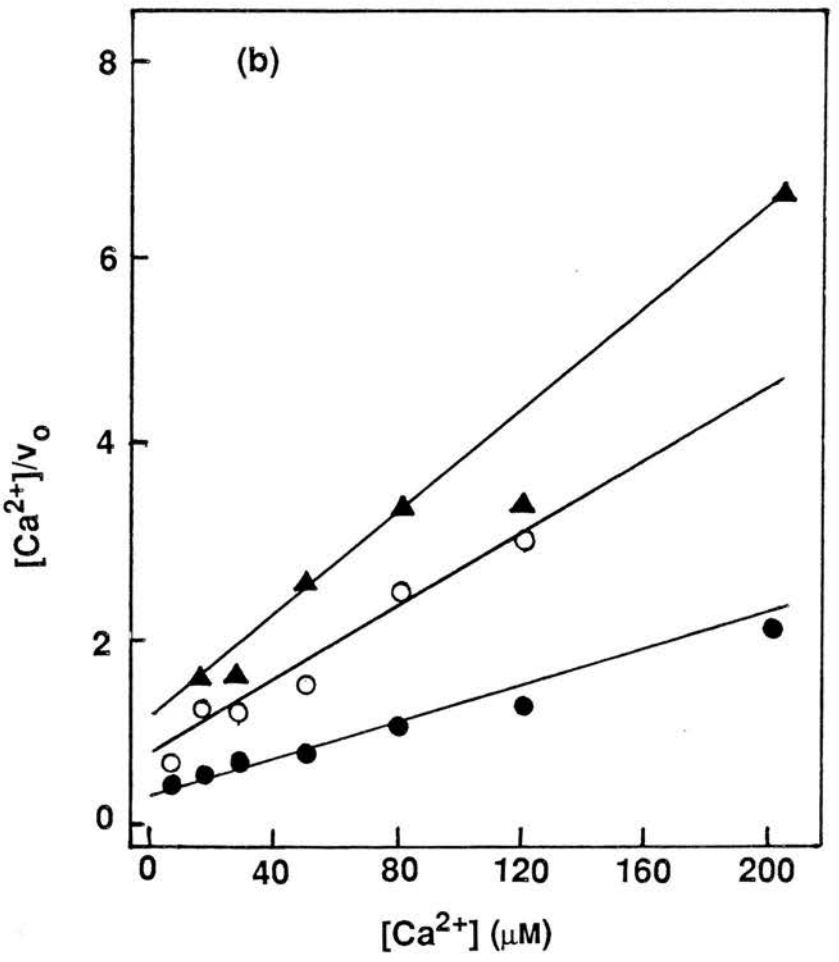
$Na^+$ addition to granules pre-incubated with $^{45}Ca^{2+}$ leads to $Ca^{2+}$ efflux. An example of this experiment is shown in Fig. 3.6. Granules were incubated with 40μM $Ca^{2+}$ for 20 min at 37°C, leading to equilibration of internal $Ca^{2+}$ with that in the medium. Addition of 20mM $Na^+$ to the medium leads to a fairly rapid efflux of $^{45}Ca^{2+}$, with a new steady state being reached after about 15 min; however, only about one-third of the accumulated $Ca^{2+}$ is released. In the presence of 100μM EGTA (tris-salt) as well as $Na^+$, a more extensive efflux occurs, with 60% of the accumulated $Ca^{2+}$ being lost after 15 min. EGTA serves to chelate the external $Ca^{2+}$ (reducing its free concentration to

***Fig. 3.4 Accumulation of $Ca^{2+}$ by granules and its inhibition by $Na^+$***

**(a)** Granules (0.16 $mg.ml^{-1}$) were incubated at 37°C in buffered sucrose containing $^{45}CaCl_2$ (0.5$\mu Ci.ml^{-1}$) and $CaCl_2$ to give the $Ca^{2+}$ concentrations shown; **(b)** as in **(a)** in the absence ( ● ) or presence of 10mM $Na^+$ (○) or 20mM $Na^+$ (▲), as $Na_2SO_4$.

$Ca^{2+}$ incorporation was assessed at 30 sec intervals. Initial rates ($v_o$) are expressed in $nmol.min^{-1}.mg^{-1}$ protein.


(a)
Initial rate (nmol.min$^{-1}$.mg$^{-1}$)
1.0
0.8
0.6
0.4
0.2
0
0
40
80
120
160
200
[Ca$^{2+}$] (μM)


(b)
[Ca$^{2+}$]/v$_o$
8
6
4
2
0
0
40
80
120
160
200
[Ca$^{2+}$] (μM)

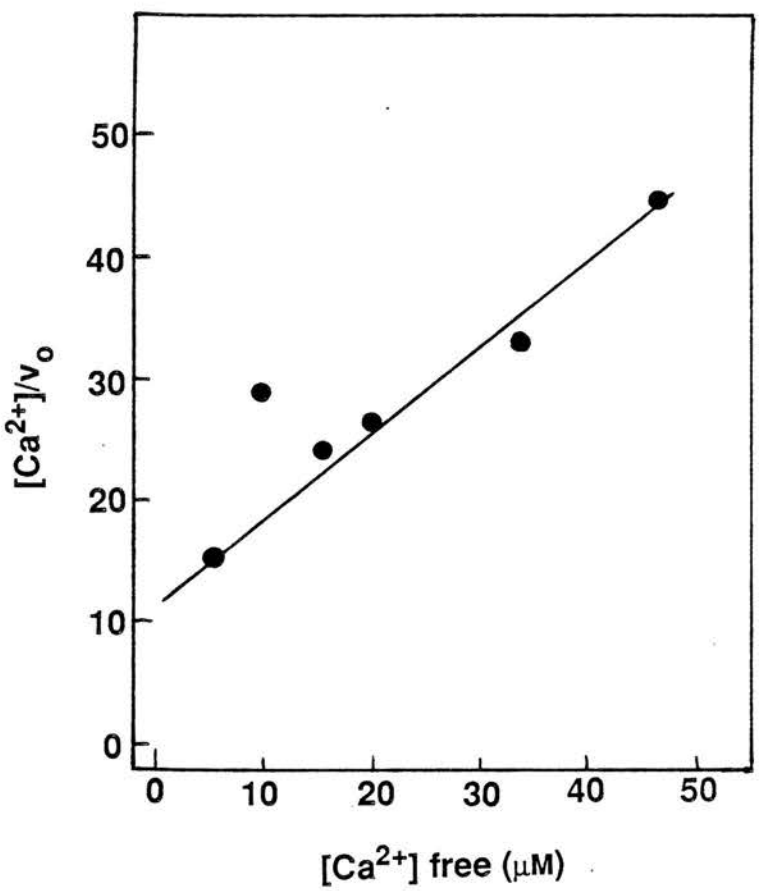



***Fig. 3.5 $Ca^{2+}$ uptake by granules in the presence of NTA***

Granules (0.3mg.ml$^{-1}$) were incubated (37°C) with buffered sucrose, pH 7.0, 1mM NTA-tris and various $CaCl_2$ concentrations to give the free $Ca^{2+}$ concentrations shown. Initial rates (nmol.mg$^{-1}$.min$^{-1}$) were assessed at 30sec intervals.

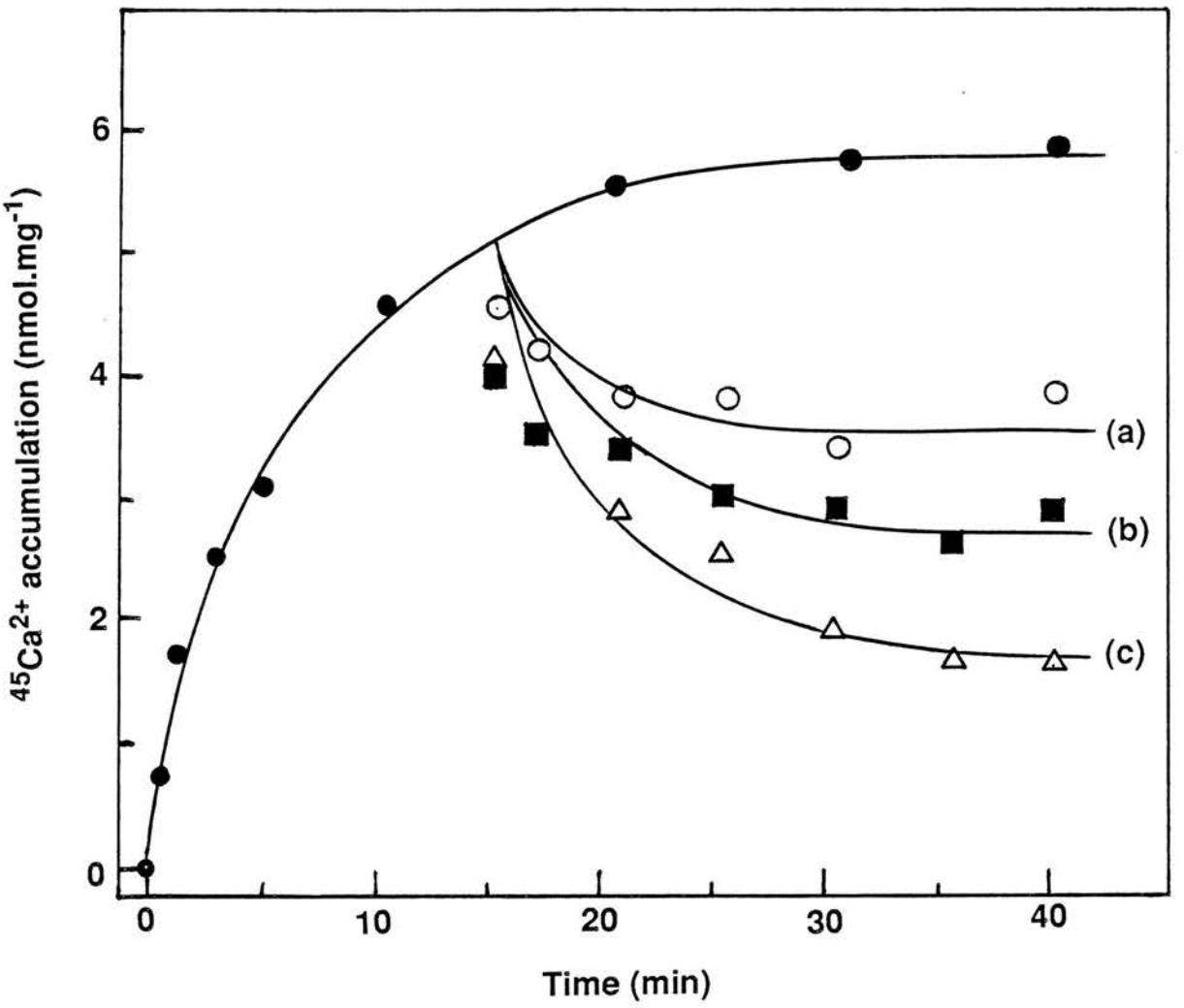



***Fig. 3.6 $Na^+$- and EGTA-induced efflux of $Ca^{2+}$ from granules***

Granules (0.3 mg.ml$^{-1}$) were incubated with 40μM $^{45}CaCl_2$ (1.0μC.ml$^{-1}$) at 37°C and $Ca^{2+}$ uptake was monitored. After 20 min the incubation was divided into three portions, and each received, either **(a)** 100μM EGTA-tris; or **(b)** 10mM $Na_2SO_4$; or **(c)** 100μM EGTA-tris plus 10mM $Na_2SO_4$. Efflux of $^{45}Ca^{2+}$ was then followed.

approximately 2nM) and EGTA alone induces the least amount of $Ca^{2+}$ release (Fig. 3.6). The mechanism of this efflux is uncertain, as in the presence of EGTA a $Ca^{2+}$ exchanging phenomenon is no longer being observed; presumably some new equilibrium condition is reached. However, the enhancement of efflux by $Na^{+}$ in the absence of external $Ca^{2+}$ (i.e., when EGTA is also present) and also the induction of release by addition of $Na^{+}$, is suggestive of a $Na^{+}/Ca^{2+}$ exchange process occuring across the granule membrane.

This was investigated further by depleting $Ca^{2+}$ from chromaffin granules using $Na^{+}$ and EGTA and then investigating the re-uptake of $Ca^{2+}$. In the control experiment shown in Fig. 3.7, chromaffin granules were preincubated in buffered sucrose medium containing non-radioactive $Ca^{2+}$. After 25 min the medium was diluted 10-fold into an identical medium containing $^{45}Ca^{2+}$, and $Ca^{2+}$ uptake was monitored (i.e. conditions similar to those in Fig. 3.3). It can be seen that $Ca^{2+}$ uptake is very similar to that shown in Fig. 3.3, i.e. a $Ca^{2+}/Ca^{2+}$ exchange process. In the other experiment shown in Fig. 3.7, granules were pre-incubated in the absence of radioactive or non-radioactive $Ca^{2+}$, but in the presence of $Na^{+}$ and EGTA; after 25 min they were then diluted into a $^{45}Ca^{2+}$-containing medium as for the control experiment. In this case, the subsequent $Ca^{2+}$ uptake is faster and reaches a higher plateau value than the control experiment. This result suggests that re-uptake was due to exchange of $Ca^{2+}$ for $Na^{+}$, the latter having entered the granule matrix by exchange for the internal $Ca^{2+}$ during the depletion with EGTA. The efflux of $Ca^{2+}$ accounts for the inhibitory effect of $Na^{+}$ on $Ca^{2+}$ uptake shown in Fig. 3.4.

It should be noted however, that $Na^{+}$ and EGTA do not induce total efflux of accumulated $Ca^{2+}$ from the granule matrix. The reason for this is unknown.

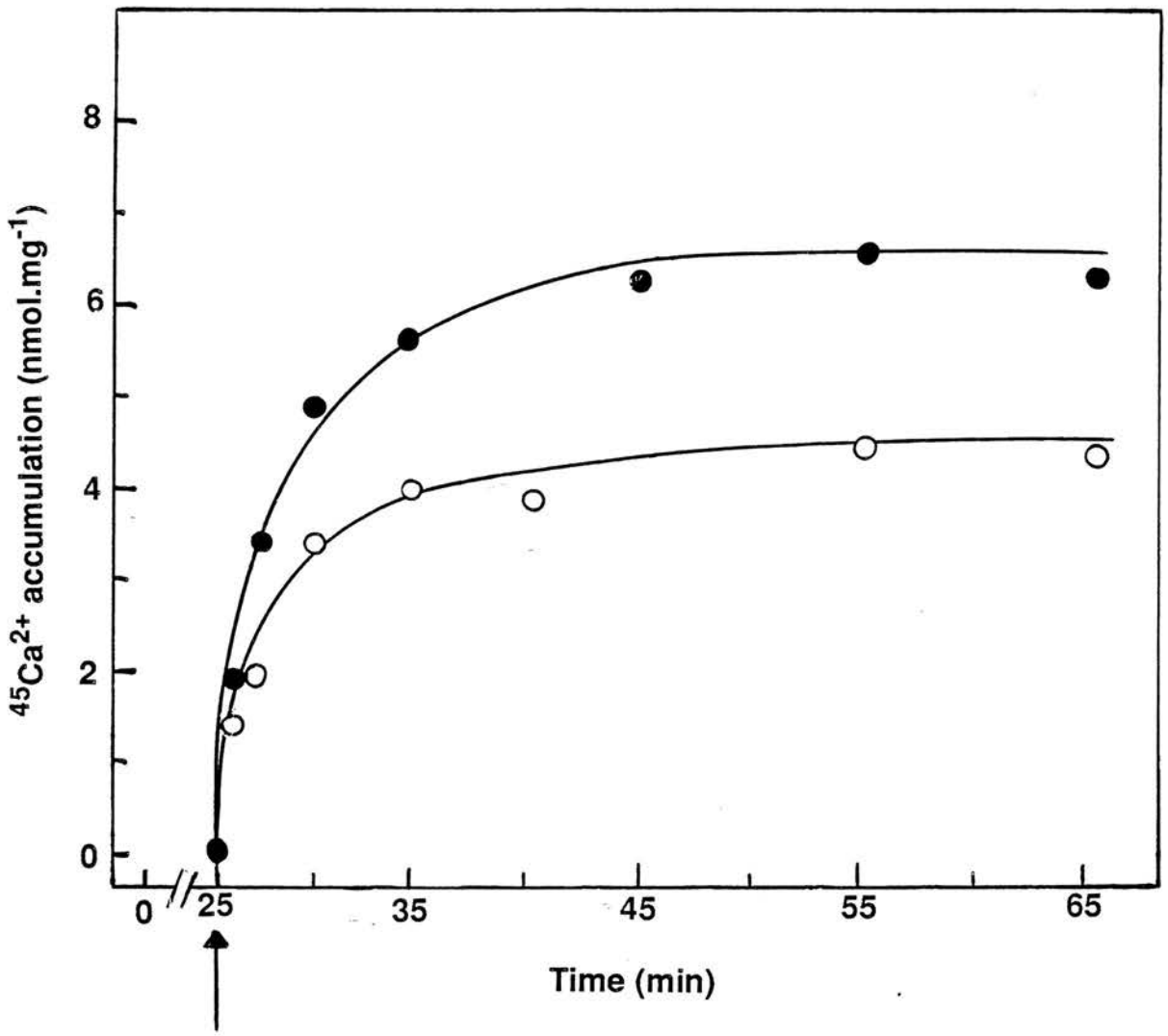



***Fig. 3.7 Uptake of $Ca^{2+}$ after $Na^{+}$ and EGTA pre-treatment***

Granules were pre-incubated at 37°C in media containing either 40μM $CaCl_2$ (○) or 20mM $Na^{+}$ plus 20μM EGTA-tris (●); no $^{45}Ca^{2+}$ was present in either medium. After 25 min (arrow) each medium was diluted 10-fold into one containing 40μM $^{45}CaCl_2$ (○,●) and $Ca^{2+}$ uptake was assessed after filtration of 100μl aliquots.

### *3.2.5 Effect of ATP on $Ca^{2+}$ Uptake*

Chromaffin granules possess an inwardly directed $H^+$-translocating ATPase which acidifies the matrix on hydrolysis of cytosolic ATP and generates an inside-positive membrane potential; the potential is dissipated by inclusion of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). $^{45}Ca^{2+}$ uptake was measured in the presence or absence of MgATP with the same concentration of free $Ca^{2+}$ in the incubation medium in both cases (Fig. 3.8). Uptake of $^{45}Ca^{2+}$ was not stimulated by MgATP. In fact, it proved to be slightly inhibitory; this may have been due to the presence of approximately 7mM $Na^+$ in the medium as the disodium salt of ATP was used. The presence of FCCP in the absence of MgATP was also without effect on the uptake. Thus, it appears that $Ca^{2+}/Na^+$ exchange is probably electroneutral, i.e. catalysing $Ca^{2+}/2Na^+$ antiport, in agreement with the results of Kostron *et al.,* (1977) and Krieger-Brauer & Gratzl (1982). There is no evidence for a direct coupling of ATP hydrolysis to $Ca^{2+}$ transport (as suggested by Burger, 1984) or of direct coupling of transport to the proton gradient.

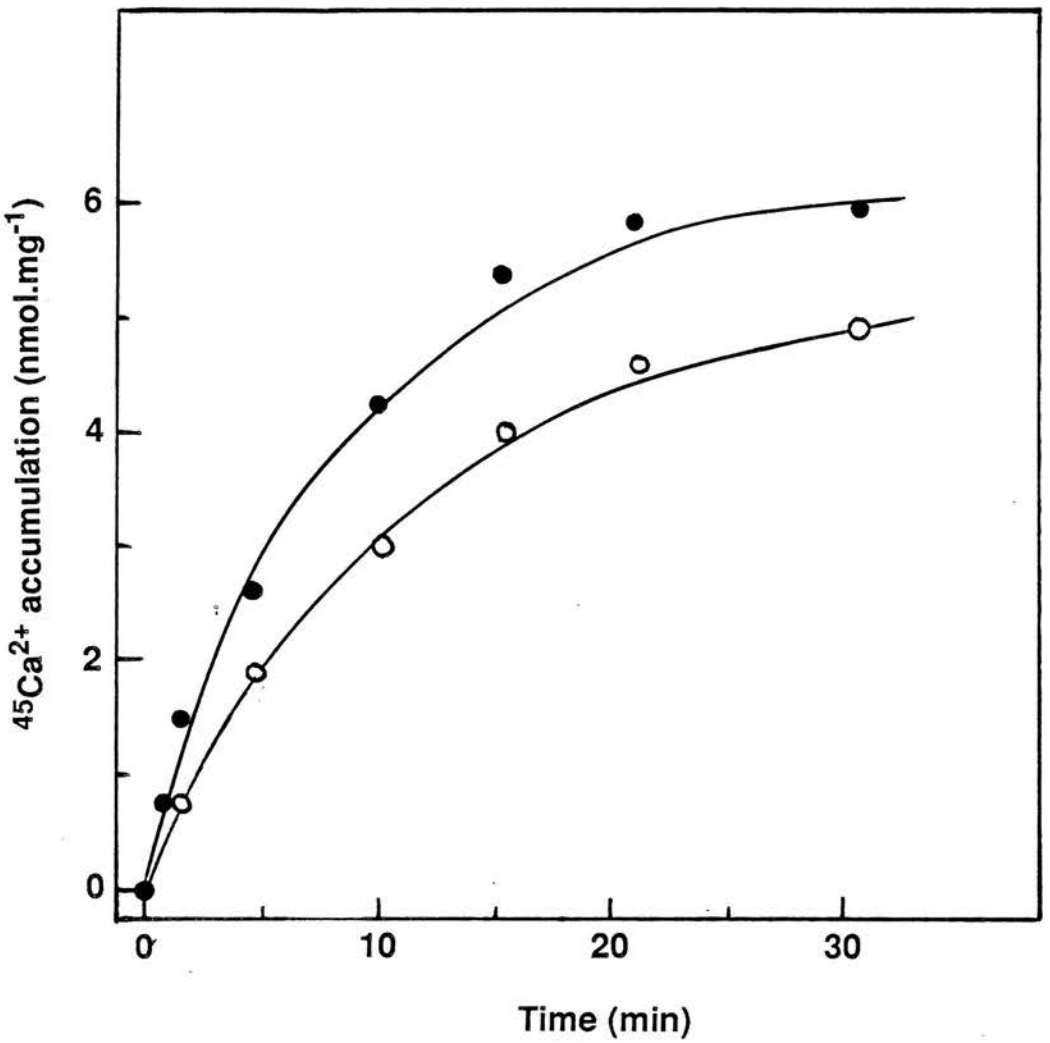



***Fig. 3.8 Effect of MgATP on $Ca^{2+}$ uptake by granules***

Granules were incubated (37°C) in buffered sucrose with (○) or without (●) 2mM ATP and 2mM $Mg^{2+}$. The $Ca^{2+}$ concentration was 40μM in both cases.

### *3.3 Discussion*

The results presented in this chapter demonstrate that purified chromaffin granules *in vitro* can be used for the study of $Ca^{2+}$ transport. Granules were purified on an iso-osmotic discontinuous sucrose-Percoll gradient and the granule fraction was collected as a band of material rather than as a pellet, as used in other studies (Krieger-Brauer & Gratzl, 1982). In addition, no attempt was made to remove Percoll from the purified granules; most incubation media, therefore, contained about 5% Percoll, although this did not affect rates of calcium transport. The total catecholamine content of the granules used in this study was 2.2μmol.mg$^{-1}$ protein and the mitochondrial contamination was very low, as assessed by cytochrome *c* oxidase activity measurements. The main drawback in using intact granules is their tendency to lyse and leak matrix constituents during incubations; in addition, their high endogenous content of catecholamines, nucleotides and ions renders transport experiments difficult because of the possibility of exchange processes occuring across the membrane.

Notwithstanding these problems, I have shown here that the granule membrane posseses a $Ca^{2+}/Ca^{2+}$ exchange activity that displays a high non-physiological $K_m$ (37 μM) when unbuffered $Ca^{2+}$ solutions were used. $Na^+$ inhibits this exchange non-competitively with a $K_i$ of 7.8mM. However, initial rates were hard to determine accurately but using $Ca^{2+}$/NTA buffered solutions, a $K_m$ of 13μM was found, similar to the value found by Crompton *et al.*, (1977) for the mitochondrial $Ca^{2+}/Ca^{2+}$ exchanger. $Na^+$ probably inhibits $Ca^{2+}$ exchange by being transported into the matrix in exchange for intragranular $Ca^{2+}$, as shown by the efflux of preloaded $^{45}Ca^{2+}$ caused by external $Na^+$ (Fig. 3.6). When granules are partially depleted of internal $Ca^{2+}$ using $Na^+$ and EGTA, subsequent addition of $^{45}Ca^{2+}$ leads to a greater uptake than occurs during simple $Ca^{2+}/Ca^{2+}$ exchange, consistent with $Na^+$ having entered the granule during the depletion phase. The kinetics of $Ca^{2+}$ exchange and its inhibition by $Na^+$, are almost identical to those observed previously in resealed membrane "ghosts" (Phillips, 1981) in which transport is uncomplicated by the presence of endogenous components.

Similarly, uptake was not stimulated by MgATP, suggesting that $Ca^{2+}$ transport by $Ca^{2+}/Na^{+}$ exchange is probably not electrogenic and that the stoichiometry is $Ca^{2+}/2Na^{+}$; however, in "ghosts", the sigmoidal relationship between $Na^{+}$ concentration and $Ca^{2+}$ flux rates that would be expected for a $Ca^{2+}/2Na^{+}$ antiport was not observed (Phillips, 1981); this was probably because initial rates could not be measured accurately.

In these experiments it is difficult to compare the magnitude of the $Ca^{2+}$ and $Na^{+}$ gradients across the membrane at equilibrium, because the concentrations of free ions within the matrix are unknown. The granules used here are prepared in sucrose that is buffered with Hepes-tris, and have not been exposed to external $Na^{+}$ or $K^{+}$ during purification. It is likely that the free concentrations of $Na^{+}$ and $Ca^{2+}$ are similar to those measured accurately in granules isolated in sucrose/Hepes-TMA, as described in Chapter 6. It is possible, though, that some matrix $Na^{+}$ and $Ca^{2+}$ has been lost during granule purification through Percoll. Intact chromaffin granules nominally contain about 20mM total matrix $Ca^{2+}$ (Phillips *et al.*, 1977). The plateau value of $^{45}Ca^{2+}$ accumulated in Fig. 3.3 (6nmol.mg$^{-1}$ protein) is equivalent to an internal concentration of 1.4mM $Ca^{2+}$, assuming the internal volume of granules is 4.3µl.mg$^{-1}$ protein (Pollard *et al.*, 1976; Phillips *et al.*, 1977). Thus, it is apparent that $Ca^{2+}$ exchange is not just limited to the "free" pool within the matrix (which is less than 10µM; see Chapter 6).

It is possible that some net uptake is occurring in these experiments but this is almost impossible to demonstrate with intact granules, since they are constantly losing their soluble constituents, including calcium, during incubations.

To date, no work has been done on $Na^{+}$ transport by intact chromaffin granules, although a net uptake of $Ca^{2+}$ would presumably be dependent on intragranular $Na^{+}$. Therefore, an additional $Na^{+}$ entry mechanism that is independent of $Ca^{2+}$ would be required. Indeed, this was suggested by the work of Phillips (1981), using resealed membrane "ghosts".

Measurements of $Ca^{2+}$ transport are very similar in intact chromaffin granules (this study) and in resealed chromaffin granule "ghosts" (Phillips, 1981; Krieger-

Brauer & Gratzl, 1983); however, the purified granules used in this study are not suitable for a detailed study of $Na^+$ transport across the granule membrane because of the disadvantages outlined above. These problems have been circumvented to a certain extent by using granule "ghosts", as described in the following two chapters.

*Chapter Four*

# *A $Na^+/H^+$ Antiporter in the Chromaffin Granule Membrane*

## *4.1 Introduction*

The pH of the cytoplasm of most eukaryotic cells is strictly maintained within the range 7.0-7.4 by several ion transport mechanisms and the high buffering capacity of the cytosol. If protons were passively equilibrated across the plasma membrane according to their electrochemical gradient, the Nernst equation predicts (with a membrane potential of -59mV and extracellular pH ($pH_o$) of 7.4), an intracellular pH ($pH_i$) of 6.4. The higher $pH_i$ (7.0-7.4) actually observed in many cell types indicates that acid equivalents are removed from the cytosol. Many physiological functions depend on $pH_i$ being maintained within these narrow limits. For example, the activity of a large number of enzymes, protein synthesis, ion conductivity, muscle contraction and cell growth and division are all shown to be increased by increases in $pH_i$ within the physiological range. Two or more pH-regulating mechanisms are present in the plasma membranes of most eukaryotic cells: the $Na^+/H^+$ antiporter is generally agreed to be universal, and anion antiport (involving $Cl^-$ and $HCO_3^-$) has also been described in many cells. In addition, $Na^+/HCO_3^-$ symport has been documented in cells involved in the transepithelial transport of acid, and $H^+$-translocating ATPases present in plasma membranes and intracellular organelles are also important in $H^+$ extrusion from the cytosol. Here, I consider the $Na^+/H^+$ antiporter, and the review by Madshus (1988) should be consulted for a discussion of the other mechanisms.

$Na^+/H^+$ antiport was first demonstrated in brush border plasma membrane vesicles of kidney and small intestine (Murer *et al.*, 1976) and it is now clear that the plasma membranes of all animal cells contain an electroneutral $Na^+/H^+$ antiporter with a stoichiometry of 1:1. Following an acidification of the cytosol, the $Na^+/H^+$ antiporter extrudes $H^+$ in exchange with $Na^+$ to restore the pH. The $Na^+$ ions that enter the cell are then pumped out by the plasma membrane $Na^+,K^+$-ATPase. Protons will enter or leave the cell via the $Na^+/H^+$ exchanger depending on the size

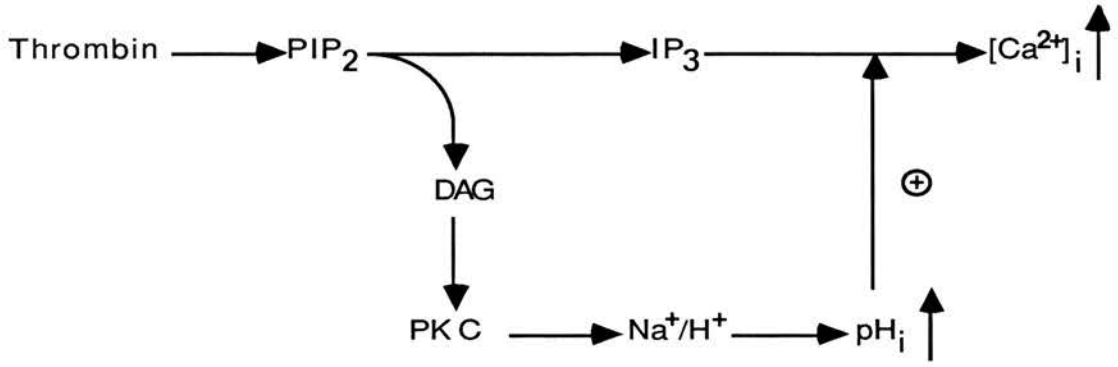
and direction of the $Na^+$ gradient. Thus, reducing the extracellular $Na^+$ concentration below the cytosolic concentration, i.e. reversal of the $Na^+$ gradient, leads to acidification of the cytosol as the $Na^+/H^+$ antiporter is activated (Moolenaar *et al.*, 1983).

Under normal conditions $[Na^+]_o/[Na+]_i \approx 10$, with $pH_i$ similar to $pH_o$, so that the antiporter operates in the $Na^+{}_o/H^+{}_i$ mode. The antiporter is inhibited competitively by the diuretic amiloride and its analogues (Lazdunkski *et al.*, 1985); these drugs and other cations, such as $Li^+$, bind to the external $Na^+$ binding site (Aronson, 1985). On the cytoplasmic face there are two separate and functionally independent $H^+$ binding sites, one of which is postulated to be an allosteric "modifier" site responsible for regulating the activity of the antiporter depending on the $pH_i$. Below a $pH_i$ of about 7.1 (the "set point"), protonation of this site activates the exchange markedly, but above this $pH_i$ the modifier site is deprotonated and very little $Na^+/H^+$ antiport occurs.

Many normal physiological responses, such as cell growth and proliferation, cell volume regulation and stimulus-secretion coupling, as well as a number of pathophysiological states such as myocardial ischaemia and hypertension, are known to be mediated, at least in part, by changes in the activity of the $Na^+/H^+$ antiporter. In most cases, the antiporter is regulated by shifting the $pH_i$-dependence of activation to a more alkaline value (i.e. raising $pH_i$ until a new "set point" is reached). For example, growth factors in serum activate $Na^+/H^+$ exchange in human fibroblasts, as does the phorbol ester 12-O-tetradecanoyl phorbol-13 acetate (TPA) in T-lymphocytes and thrombin in platelets; $Na^+/H^+$ antiport is stimulated as $pH_i$ rises by 0.15 to 0.2 units. Hormone and growth factor activation of exchange is believed to be mediated by the action of protein kinases. Protein phosphorylation may activate the antiporter directly, or may activate other proteins that are targets for the kinases.

In human platelets activation of the $Na^+/H^+$ antiporter by low concentrations of thrombin induces $Ca^{2+}$ mobilisation from intracellular stores as a result of

phosphatidyl inositol 4, 5 bisphosphate hydrolysis and diacylglycerol formation. The subsequent rise in the intracellular free $Ca^{2+}$ concentration is mediated by protein kinase C and Siffert & Akkerman (1988) have proposed the following sequence of events:




Apparently, at low thrombin concentrations, the low amounts of $IP_3$ formed would provide only a weak trigger for $Ca^{2+}$ mobilisation. Maximal alkalinisation induced by high thrombin concentrations would stimulate rapid formation of high amounts of $IP_3$, providing enough stimulation for $Ca^{2+}$ mobilisation, which then occurs independently from changes in $pH_i$ (Siffert & Akkerman, 1988). However, even in the same cell, different agonists may not work in the same way. For example, cytosolic alkalinisation induced by activation of $Na^+/H^+$ antiport following addition of adrenaline to platelets results in phospholipase $A_2$ activation and thromboxane $A_2$ formation; protein kinase C is apparently not activated. These prostanoids bind to specific receptors and activate phospholipase C, which, in turn, induces the formation of inositol phospholipids (Sweatt *et al.*, 1986; Banga *et al.*, 1986). Although the precise details have yet to be fully elucidated, it is clear that increased $Na^+/H^+$ antiporter activity has important consequences for many cell functions.

Much interesting work has been done on the activation of the $Na^+/H^+$ antiporter in the heart during recovery from myocardial ischaemia, which occurs naturally as a result of reduced coronary blood flow, or which can be induced experimentally following the ligation of the coronary arteries. Cellular ATP falls to a very low level

and there is a marked acidification of intracellular and extracellular milieus. Under these conditions the $Na^+/H^+$ antiporter is nearly inactive (Lazdunski *et al.*, 1985); $Na^+$ efflux is also prevented as a result of diminished activity of the plasma membrane $Na^+,K^+$-ATPase. When the blood flow is restored (or during experimental reperfusion with a perfusate at neutral pH) a large pH gradient is generated across the cell membrane (acid inside) and the $Na^+/H^+$ antiporter is stimulated massively; consequently there is a large $Na^+$ influx and $H^+$ efflux as the $pH_i$ is restored (Renlund *et al.*, 1984). However, during severe ischaemia the build up of arachidonic acid to concentrations of 20-30μM (Sobel *et al.*, 1978; Van der Vusse *et al.*, 1983) inhibits the $Na^+,K^+$-ATPase and the $Na^+$ that has entered the the cell cannot be pumped out. $Na^+$ efflux then occurs in exchange for $Ca^{2+}$ on the $Na^+/Ca^{2+}$ exchanger, leading to high levels of intracellular $Ca^{2+}$ which may be damaging to the cell because of the development of arrhythmias (Renlund *et al.*, 1984).

Unfortunately amiloride, which is a competitive inhibitor of the plasma membrane $Na^+/H^+$ antiporter, is not potent or specific enough for use in reperfusion following ischaemia; therefore, highly potent derivatives, such as ethylisopropylamiloride (EIPA) with its high specificity for the $Na^+/H^+$ antiporter over other cation transporting systems, may prove to be more suitable (Lazdunski *et al.*, 1985).

The existence of a $Na^+/H^+$ antiporter in an intracellular membrane, that of liver mitochondria, was first proposed by Mitchell & Moyle (1967). In heart the antiporter is very active, with a $V_{max}$ of about 100 $nmol.min^{-1}mg^{-1}$ protein and a $K_m$ for extramitochondrial $Na^+$ of 5mM (Crompton & Heid, 1978). The activity of the antiporter can be measured by passive swelling techniques, for example using iso-osmotic acetate salts (Mitchell & Moyle, 1969; Brierley, 1976). It is difficult to measure by any other means, and its physiological relevance is still unclear.

In this Chapter I show that the bovine adrenal chromaffin granule membrane possesses an amiloride-sensitive reversible $Na^+/H^+$ antiporter with a probable

stoichiometry of 1:1. The search for this antiporter was prompted by experiments on catecholamine and $Ca^{2+}$ transport across the granule membrane done previously in this laboratory. The uptake of $Ca^{2+}$ by a $Ca^{2+}/Na^{+}$ exchange mechanism would be dependent on intragranular $Na^{+}$ and this would require an additional $Ca^{2+}$-independent $Na^{+}$ uptake mechanism. Indeed, Phillips (1981) presented evidence for $Na^{+}$ transport in addition to electroneutral $Ca^{2+}/Na^{+}$ exchange, although this was not investigated in detail. However, the activity of a $Na^{+}/H^{+}$ antiporter in chromaffin granules was ruled out as a result of the failure of "ghosts" to swell significantly in iso-osmotic sodium acetate in the absence of an ionophore (Phillips, 1977). More recently, it was noted that high concentrations of extragranular $Na^{+}$ (up to 60mM), but not extragranular $K^{+}$, caused the release of catecholamines from purified intact chromaffin granules and inhibited the rate of uptake of [$^{14}$C]5-hydroxytryptamine driven by MgATP hydrolysis (J.H.Phillips, unpublished observations). These results were suggestive of the presence of a $Na^{+}/H^{+}$ antiporter, but no direct or carefully designed experiments had been done to demonstrate this conclusively.

In these experiments I have used resealed membrane vesicles of chromaffin granule membranes ("ghosts"). Such preparations, which are largely devoid of matrix components, have been used with great success to characterise in quantitative terms the mechanisms of catecholamine and electron transport across the granule membrane; internal and external milieus can be varied at will, thus allowing concentration gradients of ions to be generated across "ghost" membranes following dilution into media of different composition.

## *4.2 Results*

### *4.2.1 Inhibition of 5-hydroxytryptamine Accumulation by $Na^+$*

Chromaffin granule "ghosts" used for catecholamine transport have commonly been prepared in solutions buffered with Hepes, using $Na^+$ as the counterion to adjust the pH. For example,10mM Hepes adjusted to pH 7.0 using NaOH contains 4mM $Na^+$, and If ATP ($Na_2$ salt) is also present in the incubation medium, the total $Na^+$ concentration can approach 30mM or more. I have found that $Na^+$ is mildly inhibitory in these transport assays. 5-Hydroxytryptamine (5HT) is an excellent substrate for the amine carrier, with a $K_m$ of 5 to 8μM (Phillips, 1974 b ; Carty *et al.*, 1985). I have reinvestigated the initial rates of its transport into "ghosts" incubated with 50μM [$^{14}$C]5HT, MgATP, and various concentrations of $Na^+$ present in the medium, as described in Chapter 2; $K^+$ was also present to maintain the osmolarity constant . As shown in Fig. 4.1, $Na^+$ proves to be mildly inhibitory: approximately 50% inhibition of the initial rate of uptake was observed at the highest $Na^+$ concentration used (40mM). Therefore, $Na^+$ appears to be acting as a weak uncoupler, and I suggest below that this results from an initial reduction in the proton gradient generated by the $H^+$-ATPase by exchange of $H^+$ with $Na^+$.

### *4.2.2 Dual Uptake of $^{22}Na^+$ and [$^3$H]5HT into "Ghosts" Driven by MgATP Hydrolysis*

The inhibition of 5HT accumulation by $Na^+$ described above suggests that the transmembrane pH gradient (ΔpH) could be used to drive accumulation of $^{22}Na^+$. In the experiment shown in Fig. 4.2, "ghosts" were incubated with MgATP, 20mM KI (to increase ΔpH at the expense of $\Delta\Psi$ ), 50μM [$^3$H]5HT and 0.1mM $^{22}NaSO_4$. The $Na^+$ concentration was kept very low in order to give a reasonable specific radioactivity

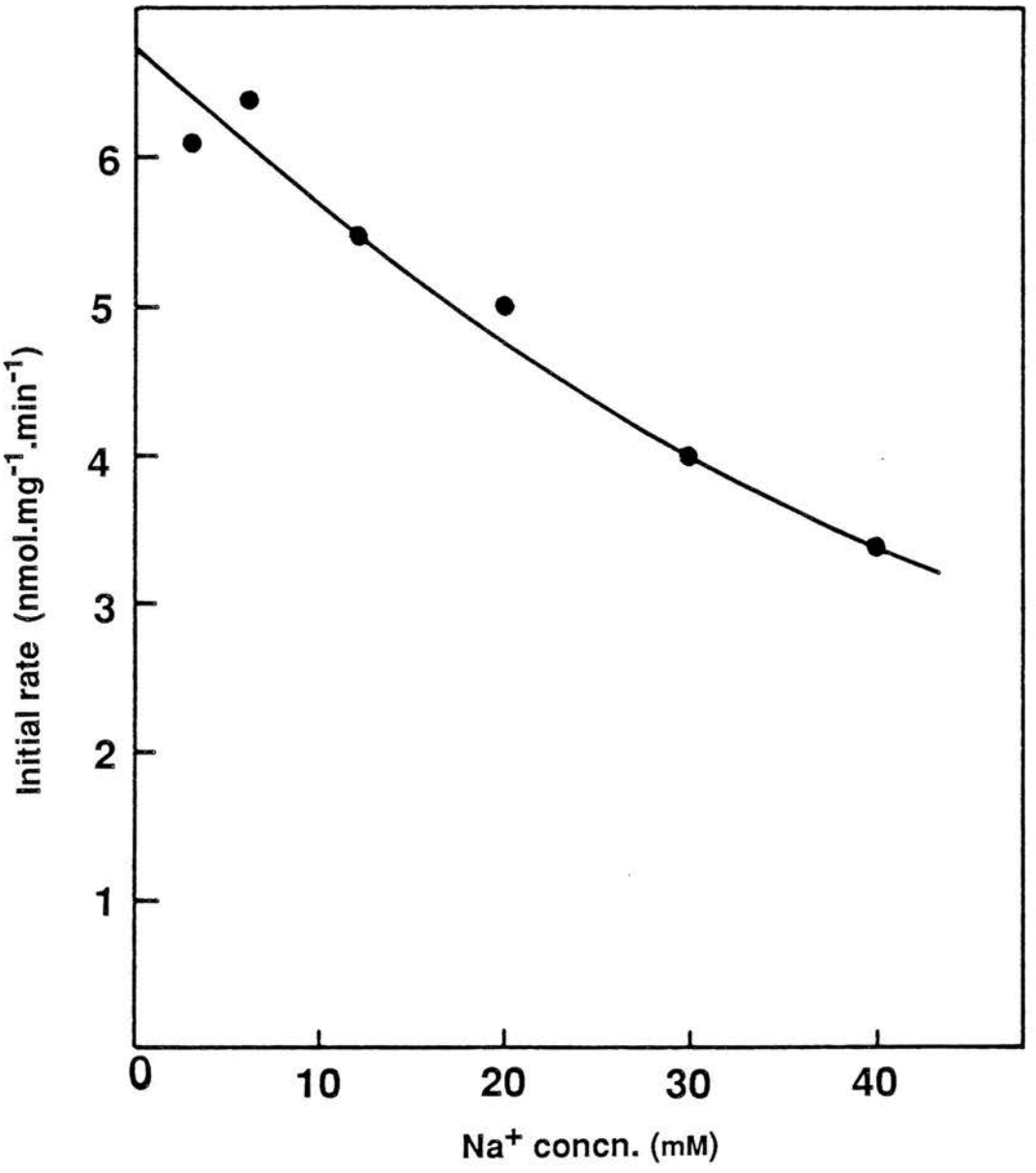



***Fig.4.1 $Na^+$ inhibition of 5HT accumulation by "ghosts"***

Chromaffin granule "ghosts" ($0.11 mg.ml^{-1}$) were incubated with 50μM [$^{14}C$]5HT and MgATP in media supplemented with various concentrations of $Na_2SO_4$; osmolarity was maintained constant by addition of $K_2SO_4$. Initial rates of accumulation of 5HT at 30°C were measured at each $Na^+$ concentration by filtering samples at intervals of 20 sec.

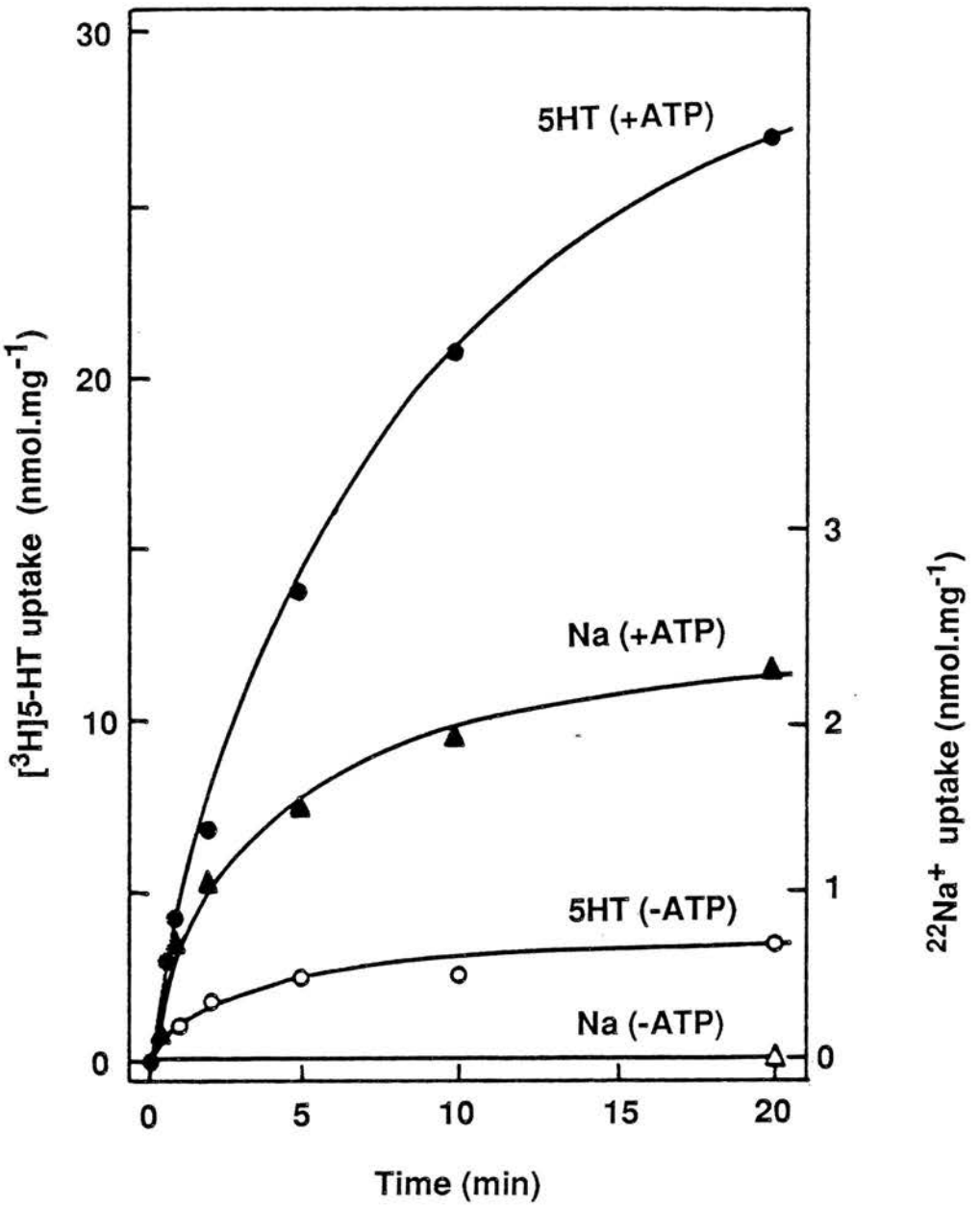



***Fig. 4.2 Accumulation of $Na^+$ and 5HT by "ghosts"***

Chromaffin granule "ghosts" (0.15mg.ml$^{-1}$) were incubated at 30°C in a standard medium supplemented with 20mM KI, 50µM [$^3$H]5HT (●,○) and 100µM $^{22}Na_2SO_4$ (▲,△). Incubations were performed in the presence (filled symbols) or absence (open symbols) of MgATP.

(15Ci/mol) so that an adequate number of radioactive counts above background could be detected in the "ghost" lumen following filtration; The $Na^+/H^+$ antiporter has a relatively high $K_m$ for $Na^+$ (at least several millimolar); at concentrations of $Na^+$ above 1mM filtration experiments are therefore difficult, the radioactive $^{22}Na^+$ being masked by non-radioactive $Na^+$ in the external medium.*

Fig. 4.2 shows that the accumulation of both substrates is dependent on MgATP. Although not shown, when samples were added to quench solution lacking sucrose, $^{22}Na^+$ and [$^3$H]5HT are released owing to the osmotic shock received by the "ghosts". This confirms that both substrates have entered the "ghost" lumen and are not just bound to their external surface. After 20 min, $Na^+$ has equilibrated across the membrane and the plateau value reached (about 2.5 nmol.mg$^{-1}$ protein) allows one to calculate the internal $Na^+$ concentration. Assuming an internal volume of 3 µl.mg$^{-1}$protein, slightly lower than the value of Phillips & Allison (1978) measured at lower osmolarity, this gives an internal $Na^+$ concentration of 0.8mM. Therefore, a concentration gradient of about 8-fold (inside over outside) has been generated across the "ghost" membrane. After 20 min 5HT is still being actively accumulated, the value of 27.5 nmol.mg$^{-1}$ at this time corresponding to an internal concentration of about 10mM; a concentration gradient of about 200-fold (inside over outside) has therefore been generated. The electrogenic catecholamine/$H^+$ exchange, responsible for the high amine concentration gradient achieved (Njus *et al.*,1987a) comes into equilibrium with the proton electrochemical gradient more slowly than the $Na^+/H^+$ exchange.

The effects of several inhibitors of the two transport systems were examined and the results are shown in Table 4.1. Because of the low amounts of $Na^+$ accumulated with only 0.1mM external $Na^+$ it was not possible to measure initial rates accurately, and therefore accumulation of both substrates after 20 min is given in the table. Under control conditions (in the absence of inhibitors) 5HT uptake was 23.2 $\pm$ 1.72 nmol.mg$^{-1}$ after 20 min (S.E.M. for three different "ghost" preparations) and $Na^+$

*It was not possible to use a constant specific radioactivity for the different $Na^+$ concentrations because of high background binding to the filters at higher concentrations.

***Table 4.1. Effects of inhibitors on $Na^+$ and 5HT accumulation by "ghosts"***

"Ghosts" were incubated with [$^3$H]5HT and $^{22}Na^+$ as described in Fig. 4.2. Values given are related to control values (in the presence of ethanol if appropriate) after 20 min incubation, and are means ± S.E.M. (n = 3).

| **Addition to medium** | **% of control value** | |
|---|---|---|
| | **5HT** | **$Na^+$** |
| Ethanol (1.0%) | 111 ± 2.6 | 120 ± 9.0 |
| $(NH_4)_2SO_4$ (10mM) | 4 ± 0.7 | 31 ± 5.5 |
| FCCP (10μM) | 19 ± 1.0 | 62 ± 3.2 |
| Ouabain (1mM) | 102 ± 5.9 | 106 ± 14.2 |
| Sodium orthovandate (10μM) | 125 ± 4.7 | 109 ± 1.6 |
| N-Ethylmaleimide (30μM) | 32 ± 2.7 | 30 ± 4.6 |
| Reserpine (10μM) | 10 ± 3.0 | 90 ± 9.2 |
| Amiloride (1mM) | 105 ± 4.0 | 68 ± 12.2 |

uptake was 1.85 $\pm$ 0.29 nmol.mg$^{-1}$. These inhibitors affect the transport of 5HT and $Na^+$ in a predictable way. Dissipation of the inside positive membrane potential generated by MgATP hydrolysis by the uncoupler FCCP, and collapse of the pH gradient across the membrane by $NH_4^+$, significantly decreased the amount of $Na^+$ and 5HT accumulated. N-ethylmaleimide (NEM), an alkylating reagent that inhibits the $H^+$-translocating ATPase of chromaffin granule membranes by binding to the 70 KDa ATP-binding subunit (Percy & Apps, 1986) also reduced the uptake of both substrates, and by the same extent. Neither substrate was affected by ouabain or vanadate, which do not inhibit this ATPase ( Percy *et al.*, 1985).

The *Rauwolfia* alkaloid reserpine inhibited the 5HT uptake via the amine transporter very markedly but only had a slight effect on the accumulation of $Na^+$; although reserpine is a potent inhibitor of the amine transporter it also has a detergent-like effect, increasing membrane permeability (Zallakian *et al.*, 1982). This may account for the slight inhibitory effect on $Na^+$ transport. Finally, amiloride had no effect on 5HT uptake, but proved to be reasonably inhibitory for the $Na^+$ uptake.

### *4.2.3 Co-localisation of $^{22}Na^+$ and [$^3$H]5HT in "Ghosts"*

It is important to show that $^{22}Na^+$ and [$^3$H]5HT are being accumulated by the "ghosts" and not by any other contaminating organelles or membrane fragments that may have similar transport mechanisms. This was assessed by subjecting the "ghosts" (after incubation for 20 min in the presence of MgATP as shown in Fig. 4.2) to equilibrium sedimentation on a sucrose density gradient. These "ghosts" are already highly purified using a discontinuous sucrose gradient method (see Chapter 2.) so that most contaminating organelle fragments have a very similar density to the "ghosts" themselves. Fractions from the gradient were assayed for $^{22}Na^+$ and [$^3$H]5HT by filtration, and for plasma membrane acetylcholinesterase and mitochondrial

cytochrome oxidase activities; Fig. 4.3. shows that the peaks of $^{22}Na^+$ and [$^3$H]5HT are co-incident. Similar amounts of non-particulate radioactivity are found at the top of the gradient, and this probably represents carry-over from the incubation mixture (or, possibly, leakage of radioisotopes from the "ghosts" during centrifugation). However, the peaks of acetylcholinesterase and cytochrome oxidase activities are found at slightly higher densities than the "ghosts"; this dual label technique can thus be used as a sensitive co-equilibrium assay for the "ghosts", and suggests that mitochondria and plasma membrane fragments are not involved in the uptake of these substrates.

### *4.2.4 pH-Jump Experiments in the Absence of ATP*

The presence of a $Na^+/H^+$ antiporter in the granule membrane was demonstrated directly by generating $Na^+$ and $H^+$ gradients across the membrane, in the absence of ATP hydrolysis. "Ghosts" were resealed and purified in media containing 10mM Mes buffer and 0.1mM EGTA at pH 6.0. They were diluted 30- to 40-fold into media containing 20mM Hepes and 0.1mM EGTA at a more alkaline pH (pH 7.0 or pH 8.0) plus a small amount of $^{22}Na^+$. Thus, a large outwardly directed $H^+$ gradient ($H^+_{in} > H^+_{out}$) is generated (Fig. 4.4); at an external $Na^+$ concentration of 0.5mM $^{22}Na^+$ is accumulated rapidly with an approximate initial rate of 0.75 $nmol.mg^{-1}10sec^{-1}$ (Fig. 4.4b) and reaches a plateau after about 2 min that is maintained for at least 10 min. The size of the plateau value depends on the magnitude of the pH gradient imposed. As shown in Fig. 4.4b with $\Delta$pH of 1.0 and 0.5mM $Na^+$, the plateau value corresponds to an internal $Na^+$ concentration of 0.7mM; with $\Delta$pH of 2 and 1.0mM $Na^+$ the internal $Na^+$ concentration is 2.7mM (Fig. 4.4a). In the absence of a transmembrane pH gradient ($pH_i = pH_{out} = 6.0$; Fig. 4.4a) very little $Na^+$ is accumulated, reaching a value in the "ghost" lumen of about 0.2mM with 1.0mM externally.

***Fig. 4.3 Sucrose-gradient analysis of "ghosts"***

Chromaffin-granule "ghosts" were incubated with MgATP, [$^3$H]5HT and $^{22}Na_2SO_4$ as described in Fig. 4.2. After 20 min at 30°C they were analysed on a sucrose gradient. Portions of the fractions were collected, filtered and analysed for radioactivity ([$^3$H]5HT, ● ; $^{22}$Na, ▲ ) or were assayed for acetylcholinesterase ( △ ) or cytochrome *c* oxidase ( ○ ) activities. Refractive index measurements made on a parallel gradient were used for calculation of fraction densities ( □ ).

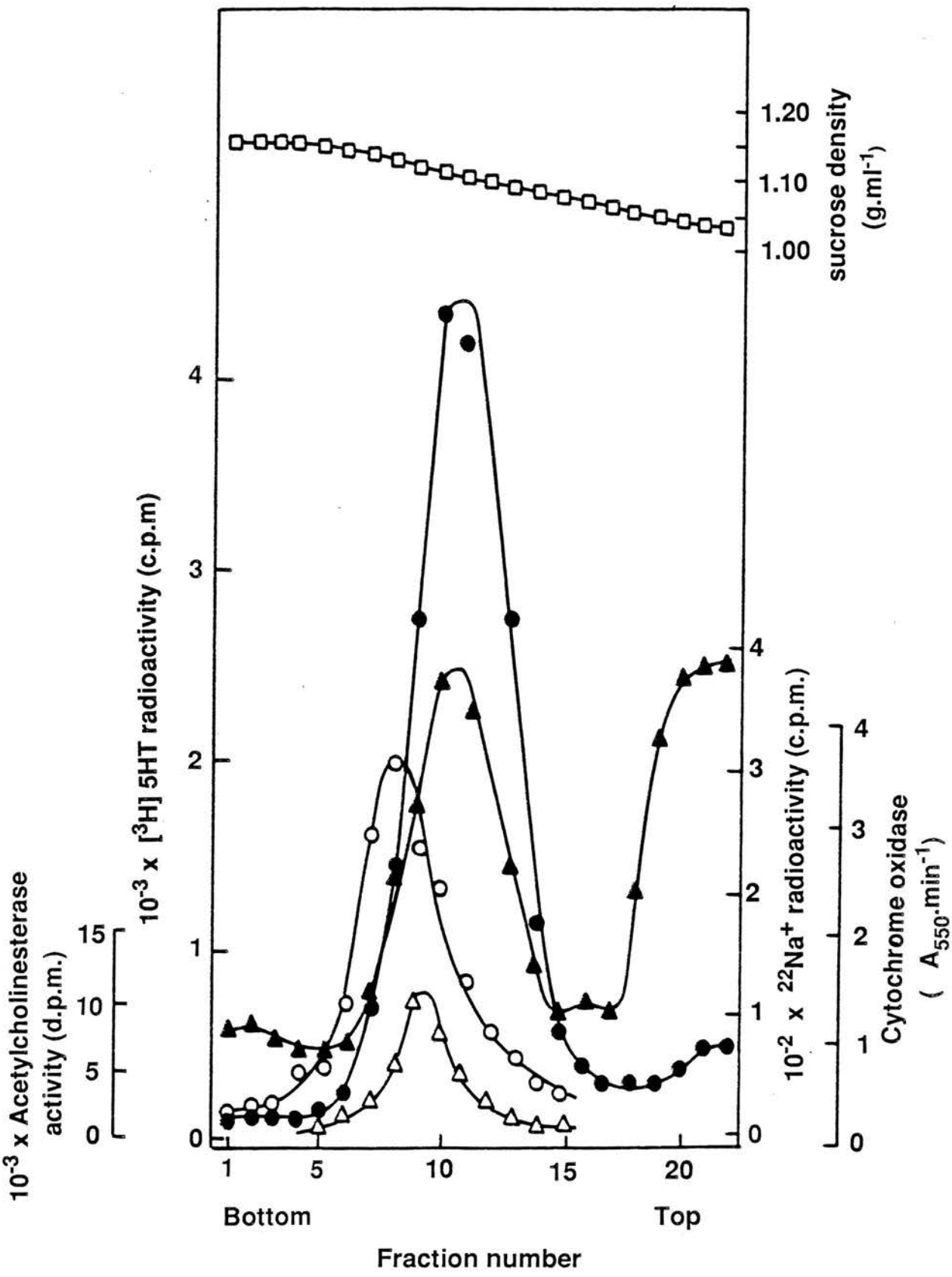
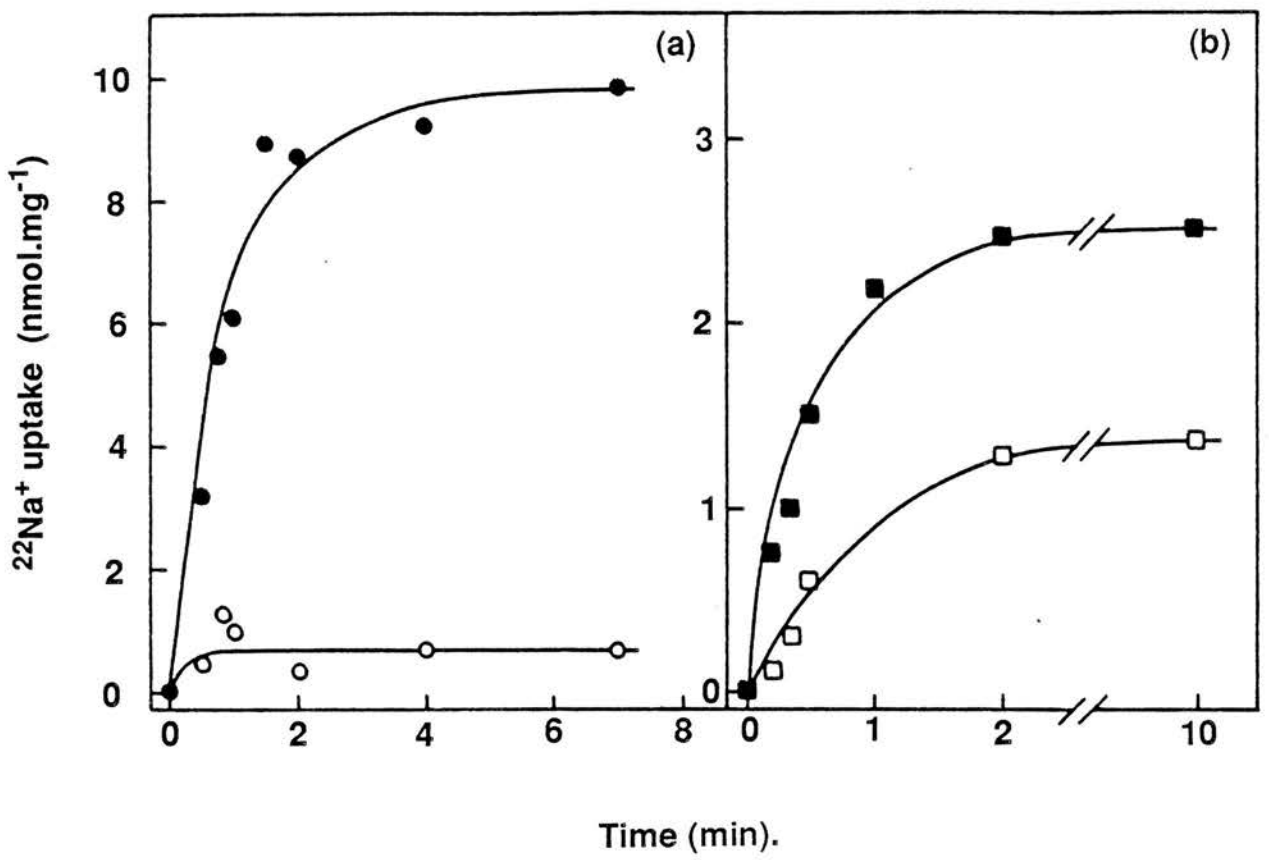
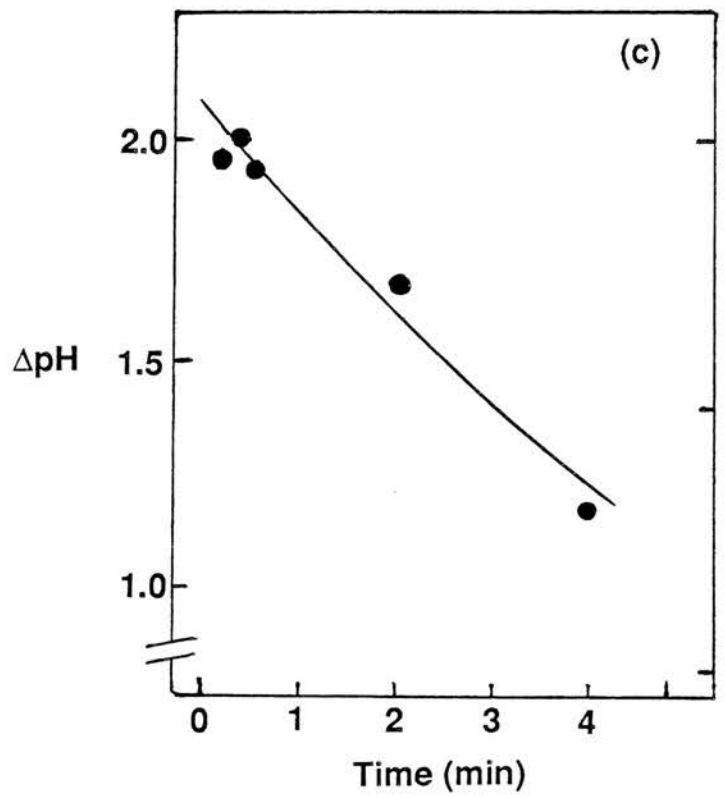

sucrose density (g.ml-1)
1.20
1.10
1.00
10-3 x [3H] 5HT radioactivity (c.p.m)
10-2 x 22Na+ radioactivity (c.p.m.)
Cytochrome oxidase ( A550.min-1)
10-3 x Acetylcholinesterase activity (d.p.m.)
Bottom
Top
Fraction number

***Fig. 4.4 Accumulation of $Na^+$ and methylamine by acid-loaded "ghosts"***

Chromaffin granule "ghosts" loaded with buffer at pH 6.0 were diluted to a concentration of 0.2 $mg.ml^{-1}$, into media at 30°C as described in section 2.3.3., Chapter 2. These media contained: **(a)** 1.0mM $^{22}NaCl$ ($4\mu Ci.ml^{-1}$) with final pH values of 8.0 (●) and 6.0 (○); **(b)** 0.5mM $^{22}NaCl$ ($4\mu Ci.ml^{-1}$) at pH 7.0, without (■) or with 1mM-amiloride (□); or **(c)** 2.5μM [$^{14}C$]methylamine ($0.15\mu Ci.ml^{-1}$) at pH 8.0


(a)
(b)
$^{22}Na^+$ uptake (nmol.mg$^{-1}$)
Time (min).


(c)
ΔpH
Time (min)

In order to compare the magnitude of the $Na^+$ and $H^+$ gradients at the plateau value of $Na^+$ reached in Fig. 4.4a, alkalinisation of the "ghost" lumen was measured following a pH jump of 2 pH units, with 1mM $Na^+$ (but no $^{22}Na^+$) and a trace amount of [$^{14}$C]methylamine in the external medium (i.e, conditions of Fig.4.4a). Although difficult to measure accurately, the pH gradient was rapidly dissipated, with a $\Delta$pH of about 1.2 being measured 4 min after transfer to the alkaline medium (Fig. 4.4c). Very little $\Delta$pH was apparent after 7 min. Thus, collapse of the pH gradient over this time period presumably results in a decreased driving force for $Na^+$ uptake, and hence the relatively small $Na^+$ gradient ($Na^+_{in} > Na^+_{out}$) that is generated across the "ghost" membrane.

I attempted to use the pH-jump method to investigate the kinetics of $Na^+$ accumulation, employing a pH-jump from pH 6.0 to pH 7.0, and $Na^+$ concentrations in the range 0.5-50mM (Fig. 4.5). The shortest incubation time that it was practicable to use however, was 10 sec, and as shown in Fig. 4.4b, the accumulation is non-linear at this point. A plot of [S]/v against [S] for the data in Fig. 4.5 gives a $K_m$ for $Na^+$ of 20-25mM with a $V_{max}$ of 150-200 nmol.min$^{-1}$.mg$^{-1}$. I was unable to make more accurate measurements than this.

### *4.2.5 $Na^+$-jump Experiments in the Absence of ATP*

If the coupled $Na^+$ for $H^+$ exchange is reversible then a transmembrane $Na^+$ gradient should be capable of driving $H^+$ transport. For this, "ghosts" resealed and purified in the presence of 25mM $Na_2SO_4$ (50mM $Na^+$) at pH 7.0 were diluted 30-fold into media at the same pH but devoid of $Na^+$; this generates a large outwardly directed $Na^+$ gradient ($Na^+_{in} > Na^+_{out}$). Proton uptake (i.e. acidification of the "ghost" matrix) was monitored by measuring the accumulation of [$^{14}$C]methylamine, a permeant weak base that is accumulated in acidic compartments. As Fig. 4.6 shows, a pH gradient (acidic inside) is established rapidly and is maintained for a considerable

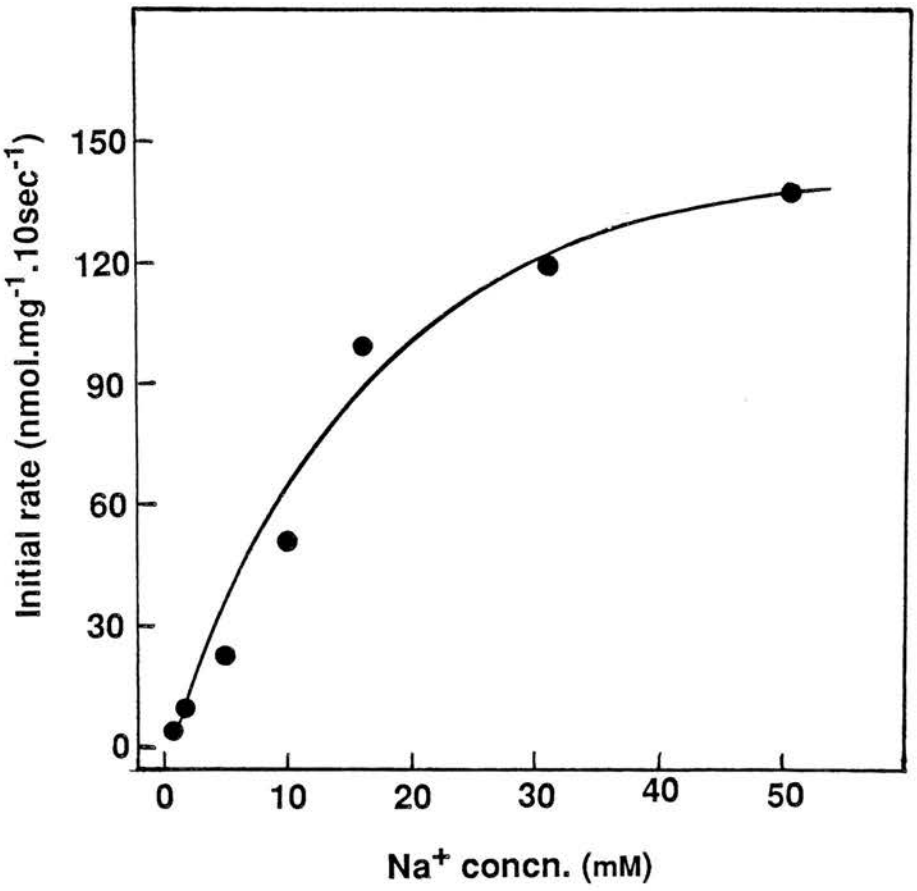



***Fig. 4.5 Uptake of $Na^+$ by "ghosts" in response to a pH-jump***

"Ghosts" (O.15mg.$ml^{-1}$) containing 10mM Mes at pH 6.O were diluted into buffered sucrose (pH 7.0) containing 0.1mM EGTA, $^{22}NaCl$ (3μCi.$ml^{-1}$) and $Na_2SO_4$ to give the $Na^+$ concentrations shown. Initial rates were assessed by terminating the uptake after 10 sec.

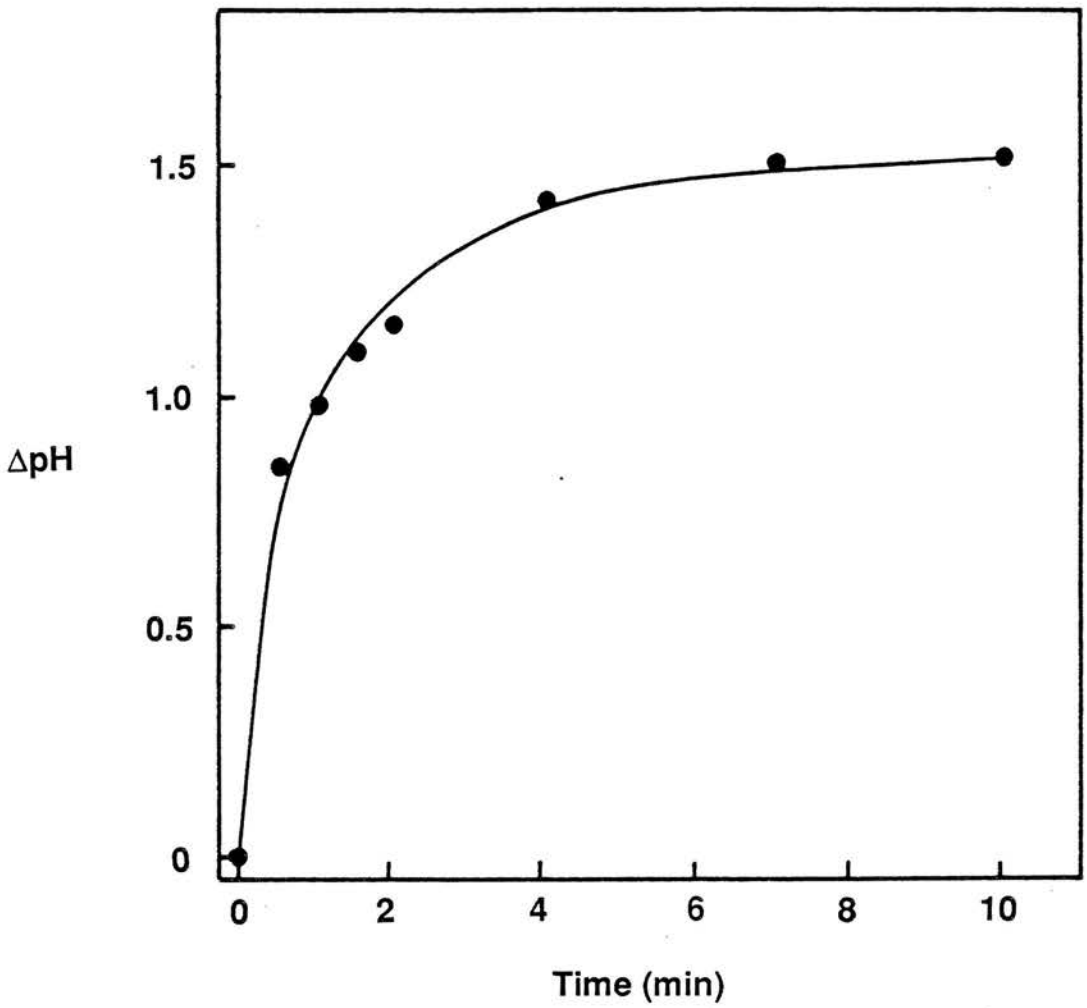



***Fig. 4.6 Generation of a pH gradient by $Na^+$-loaded "ghosts"***

Chromaffin granule "ghosts" containing 25mM $Na_2SO_4$ were diluted into a medium at 25°C to give a protein concentration of 0.12mg.ml$^{-1}$ and a final sodium concentration of 1.7mM. "Ghost" acidification was followed by measuring the accumulation of [$^{14}$C]methylamine and is plotted as the pH gradient (ΔpH) across the membrane.

time. A ΔpH of about 1pH unit is generated after 1 min and at equilibrium an apparent 30-fold concentration gradient of $Na^+$ ($Na^+_{in}$ = 25mM; $Na^+_{out}$ = 1.7mM) sustained a pH gradient of 1.5 pH units ($pH_{in}$ = 5.5; $pH_{out}$ = 7.0, equivalent to a 30-fold $H^+$ concentration gradient. This suggests a stoichiometry of exchange of 1:1 if it is the only mechanism for linking the two gradients.

### *4.2.6 Fluorescence Assay of $Na^+/H^+$ Exchange*

In addition to radioisotope methods for measuring $Na^+$ and $H^+$ fluxes, I have also used a fluorescent permeant weak base, 9-amino-6-chloro-2-methoxyacridine (ACMA) which is accumulated in acidic compartments. The fluorescence of the internal dye is quenched and is assumed not to contribute to the measured (i.e. external) fluorescence. The theory of accumulation and quenching of ACMA fluorescence is described in detail in Chapter 2. Fluorescence quenching was calibrated (Warnock *et al.*, 1982) by diluting "ghosts" resealed with 10mM Mes at pH 6.0 into media containing sucrose buffered with 30mM Mes or Hepes at various pH values ranging between 6.1 to 8.3 (adjusted using KOH or TMA hydroxide). The percentage of quenched fluorescence (%Q) is a direct measure of ACMA uptake ($A_i$), and the value (100-%Q) gives the amount of external ACMA ($A_o$).

Results were plotted according to eqn. (1)

$$\log (A_i/A_o) = \Delta pH + \log (v_i/v_o) \qquad (1)$$

where ΔpH is the transmembrane pH difference (outside-inside), and $v_i$ and $v_o$ are the intra- and extra-vesicular volumes, assumed to remain constant. Therefore, the quenching of ACMA fluorescence is dependent on the magnitude of the pH gradient, and good straight-line plots were obtained (Fig. 4.7). The measured slope was found to vary according to the cation ($K^+$ or TMA) in the medium: in Fig. 4.7a, a slope of 0.89

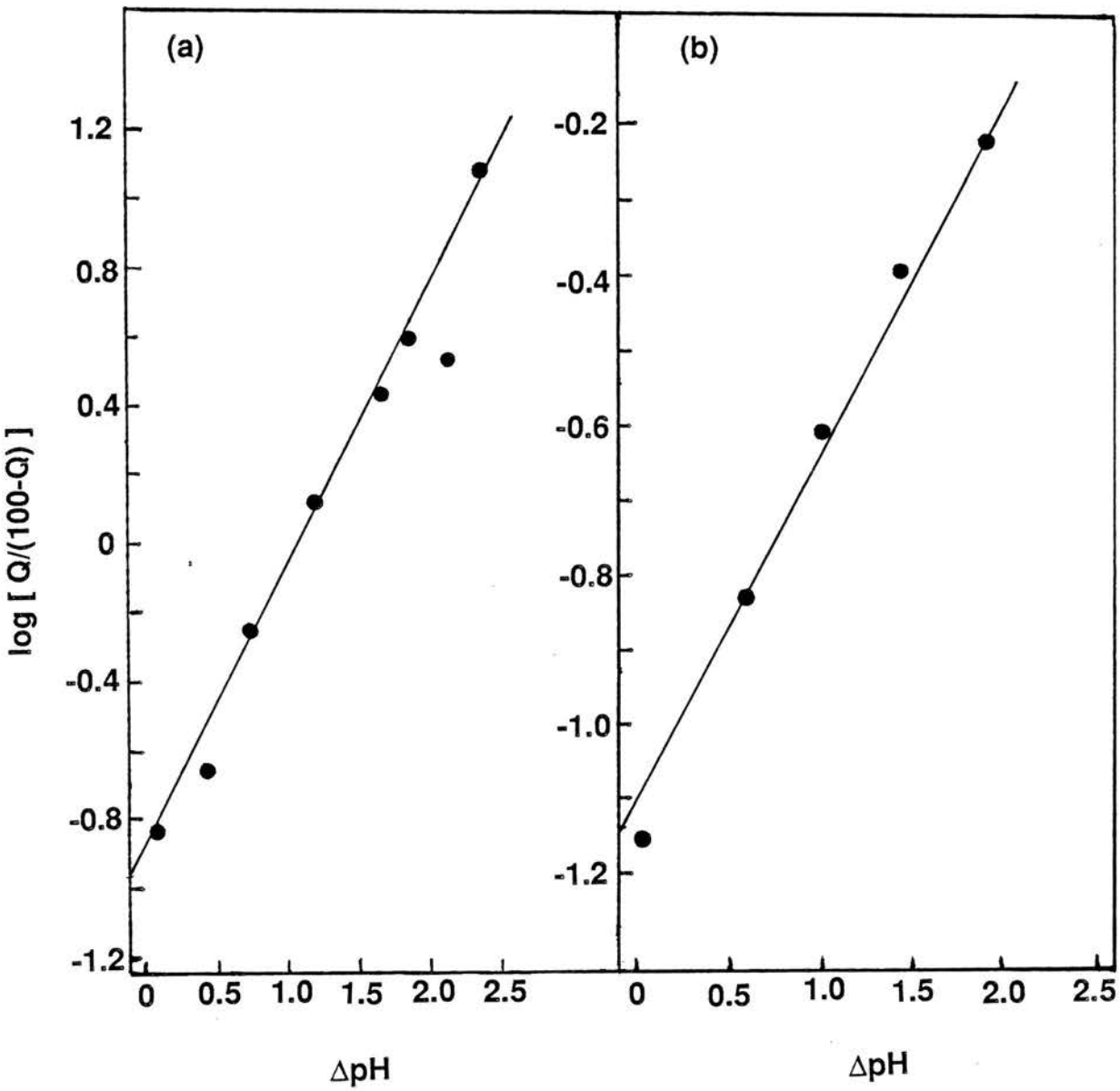



***Fig. 4.7 ACMA quenching by chromaffin granule "ghosts"***

"Ghosts" (0.1mg.ml$^{-1}$) resealed in Mes buffer at pH 6.0 were diluted into media buffered at various pH values, adjusted using either **(a)** KOH or **(b)** TMA hydroxide, and ACMA quenching (Q) was measured.

and a y-intercept of -0.97 was found with $K^+$; with TMA the slope was 0.58 with a y-intercept of -1.13 (Fig. 4.7b). The y-intercept is equal to - $\log(v_i/v_o)$ giving notional values for $(v_i/v_o)$ of 1/9.3 and 1/13.5 for $K^+$ and TMA, respectively. The actual values for this ratio are 1/2700 for $K^+$ and 1/1087 for TMA.

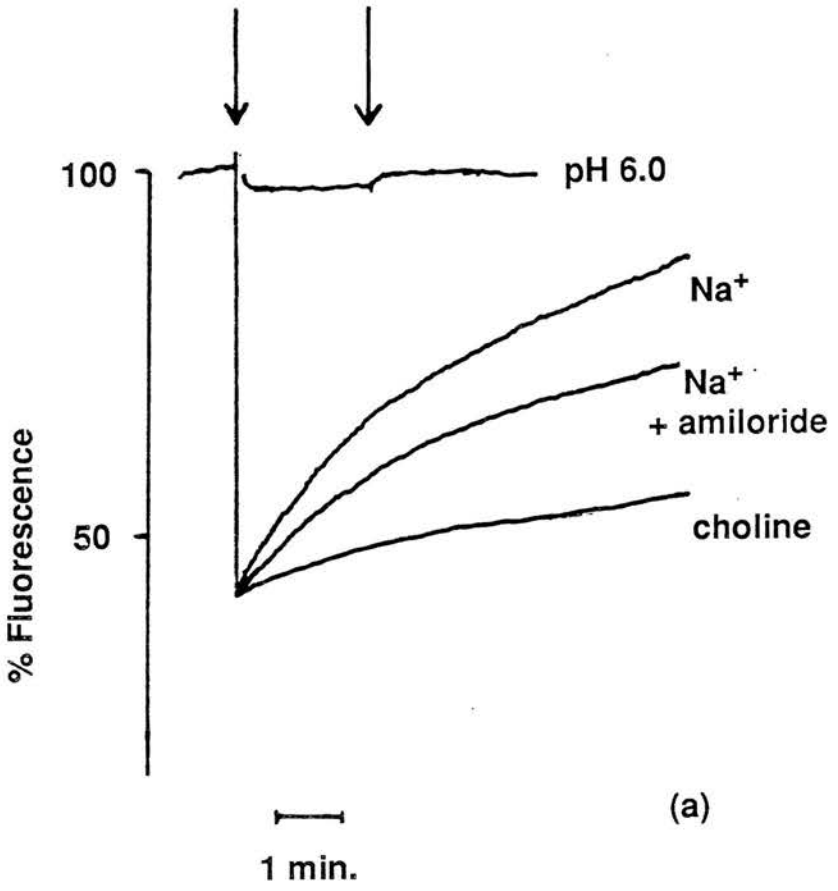
This deviation of both slope and intercept from the theoretical values has been demonstrated in gastric microsomal vesicles (Lee *et al.*, 1978) and brush border membrane vesicles of kidney (Warnock *et al.*, 1982), and is assumed to result from binding of fluorescent probe to the membranes.

When "ghosts", resealed with Mes at pH 6.0 were diluted into a medium at pH 7.0 containing ACMA, the resulting quenching was about 55% (Fig. 4.8a), corresponding to a $\Delta$pH of about 1pH unit (read from Fig. 4.7a). In the presence of choline chloride in the medium there is a very slow recovery of the quenched fluorescence, but when this is replaced by NaCl the recovery is much faster because the $\Delta$pH across the membrane is dissipated as internal $H^+$ exchanges for external $Na^+$. Under the conditions of Fig. 4.8a, with 50mM $Na^+$, the fluorescence trace returns to close to its starting value (100%) after about 10 min; the most likely explanation for the failure to return precisely to the initial fluorescence value is dye binding by the membranes (Warnock *et al.*, 1982). Using this technique I was able to measure the initial rate of recovery (arbitrary fluorescence units) of ACMA fluorescence, following a pH-jump with varying external $Na^+$ concentrations (up to 60mM) and thus derive an apparent $K_m$ for $Na^+$ during $Na^+/H^+$ antiport (Fig. 4.9). A value of 4.7 $\pm$ 0.6mM (mean $\pm$ S.E.M. for five independent determinations) was found when a pH 6.0 to pH 7.0 jump was employed.

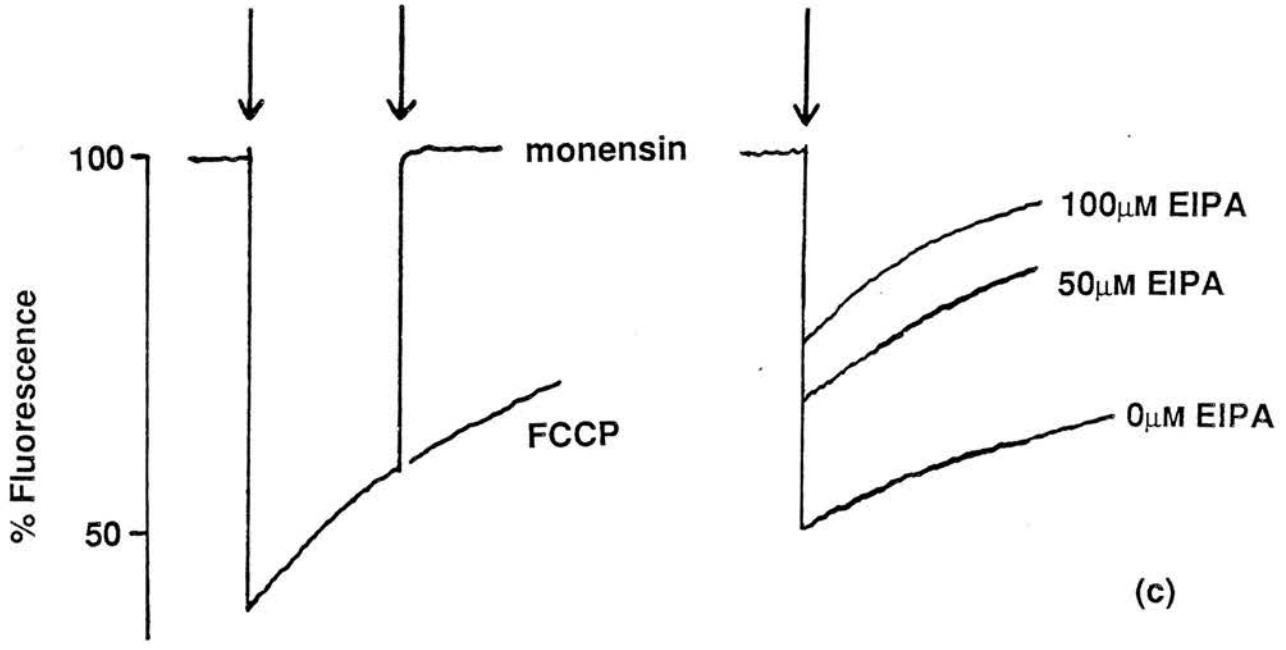
Some control experiments are shown in Figs. 4.8a and 4.8b. Lack of a pH gradient ($pH_{in}=pH_{out}=6.0$) results in no uptake and quenching of ACMA fluorescence and subsequent addition of $Na^+$ has no effect on the trace (Fig. 4.8a). However, following a pH-jump in the presence of external $Na^+$, addition of the electroneutral ionophore monensin (which catalyses rapid $Na^+/H^+$ exchange) caused immediate recovery of

***Fig. 4.8 Demonstration of $Na^+$ entry into acid-loaded "ghosts" using ACMA fluorescence***

**(a)** "Ghosts" loaded at pH 6.0 were diluted into medium at pH 7.0 (25°C) containing ACMA at the times shown by the first arrow, and fluorescence was recorded. Media were supplemented with 50mM choline chloride, 50mM NaCl, or 50mM NaCl plus 1mM amiloride. In a control experiment, the medium was at pH 6.0; 50mM NaCl was added at the second arrow. **(b)** As (a), with medium containing 50mM NaCl; at the second arrow was added either 10μM monensin or 10μM FCCP. **(c)** As (a), with the medium containing 50mM choline chloride and either 50μM or 100μM ethylisopropylamiloride (EIPA). In all cases the final protein concentration was 0.12mg.$ml^{-1}$.


100
50
% Fluorescence
pH 6.0
Na+
Na+
+ amiloride
choline
1 min.

(a)


100
50
% Fluorescence
monensin
FCCP
100μM EIPA
50μM EIPA
0μM EIPA

(b)

(c)

fluorescence to about its starting value. The electrogenic proton ionophore, FCCP, did not affect the trace, showing that it had no effect on $Na^+/H^+$ exchange. Fig. 4.8a. also shows that $Na^+$ induced $H^+$ efflux is inhibited by amiloride and the ACMA quenching technique allows one to determine some information on the nature of amiloride inhibition. As before, initial rates of recovery of ACMA fluorescence were measured over a range of external $Na^+$ concentrations but also including several concentrations of amiloride in the medium. Fig. 4.9 is a Hanes plot ([S]/v vs. [S]) and shows that amiloride is a competitive inhibitor of $Na^+/H^+$ exchange with an apparent $K_i$ of 0.26 $\pm$ 0.02mM (mean $\pm$ S.E.M. for three amiloride concentrations).

The highly specific and potent analogue of amiloride, ethylisopropylamiloride (EIPA), was also investigated. Surprisingly, low concentrations (up to 50$\mu$M) appeared to have no effect on $Na^+$ entry. When present at 20 to 100$\mu$M, EIPA decreased the extent of ACMA quenching following a pH-jump, and increased the rate of leakage of protons in the presence of choline chloride (Fig. 4.8c); it is possible that EIPA facilitates net cation/$H^+$ exchange, collapsing the pH gradient in a concentration-dependent manner. 30$\mu$M EIPA was found to decrease the initial rate of uptake of 5HT (driven by MgATP hydrolysis) by 70%, consistent with it facilitating proton efflux (results not shown). However, the sample of EIPA used here has not been tested for its potent inhibitory action on a well-known plasma membrane $Na^+/H^+$ antiporter and therefore I do not have a positive control to determine whether the sample (received as a gift) acts in the accepted manner.

I also used the ACMA quenching technique to look at the specificity of the exchanger for different monovalent cations in addition to $Na^+$. In common with many plasma membrane $Na^+/H^+$ antiporters (Aronson, 1985) $Na^+$ could not be replaced by $K^+$, but $Li^+$ was a weak alternative substrate. Although not shown, $Li^+$-driven $H^+$ efflux was demonstrated, with an apparent $K_m$ for $Li^+$ of about 6mM; however, initial rates were only about one-fifth of those found with $Na^+$.

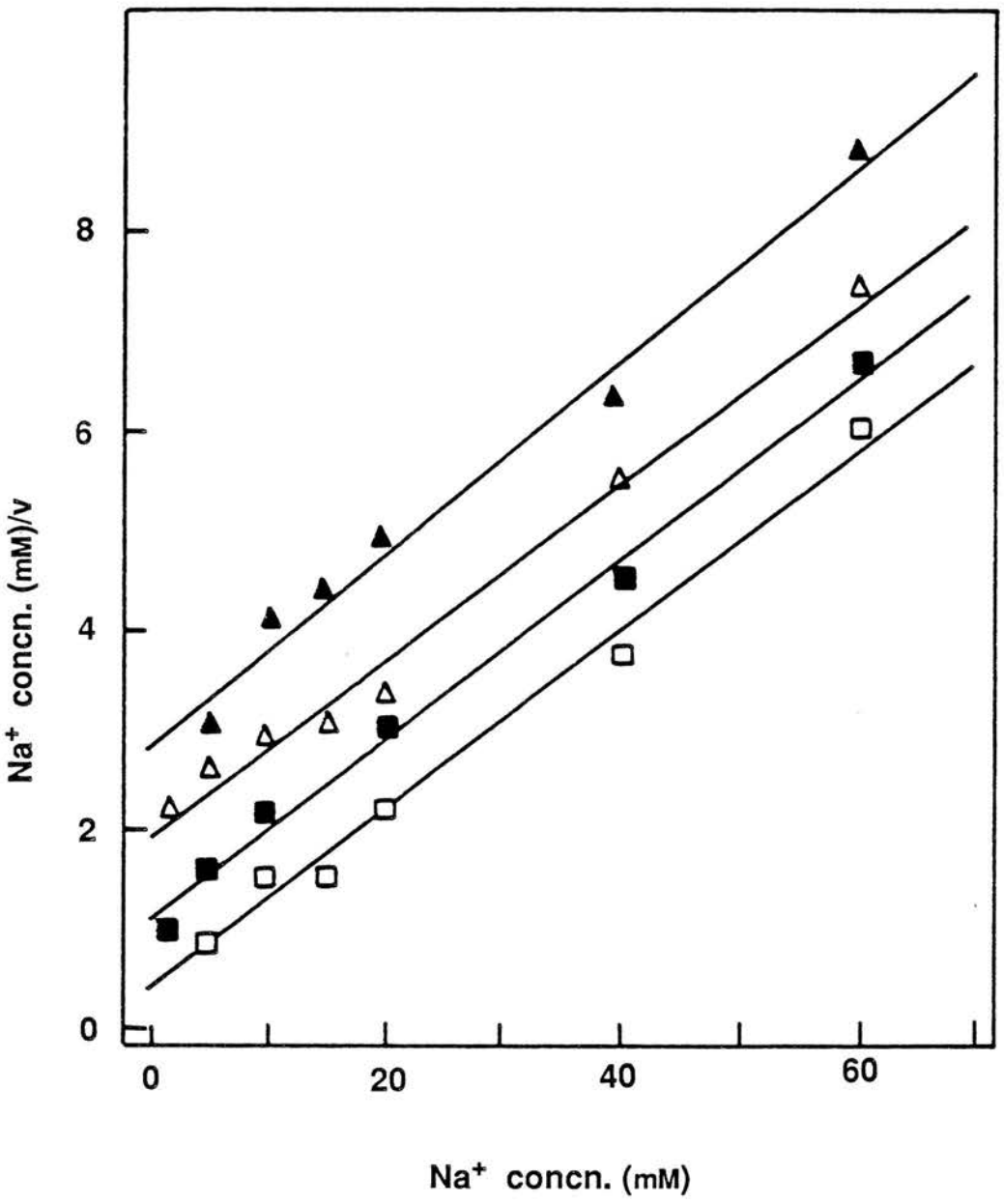



***Fig. 4.9 Competitive inhibition of $Na^+/H^+$ antiport by amiloride***

$Na^+$ entry into acid-loaded "ghosts" was assayed by ACMA fluorescence as described in Fig. 4.8. Media were supplemented with NaCl and choline chloride to make a total salt concentration of 60mM. Initial rates of recovery of ACMA fluorescence (v, arbitrary units) were measured in the absence of amiloride ( □ ), or in the presence of 0.5mM ( ■ ), 1.0mM (△), or 1.5mM ( ▲ ) amiloride.

### *4.2.7 Reversibility of the $Na^+/H^+$ Antiporter*

Reversibility of exchange was also demonstrated using the ACMA quenching technique. In Fig. 4.10 "ghosts" containing 50mM $Na^+$ at pH 7.0 were diluted 30-fold into a $Na^+$-free medium, adjusted to pH 7.0 with TMA, and $H^+$ uptake was monitored. There was an initial rapid quenching of fluorescence, followed by a slower, further acidification which reached equilibrium after 10 to 20 minutes; under these conditions the equilibrium is maintained for at least a further 30 minutes. The 30-fold gradient of $Na^+$ sustains a pH gradient of 1.5 pH units (inside acidic) as measured by the percentage of quenching of ACMA (read from Fig. 4.7b). In the absence of a transmembrane $Na^+$gradient ($Na^+_{in} = Na^+_{out}$), no ACMA quenching occurs. Comparison of Figs. 4.6 and 4.10 demonstrates a very good agreement in the size of $\Delta$pH generated across the"ghost" membranes following a $Na^+$-jump, using the two techniques of methylamine accumulation and quenching of ACMA fluorescence. After 30 min, addition of either monensin or FCCP has no effect on the fluorescence trace shown in Fig. 4.10, demonstrating that the $Na^+$ and $H^+$ gradients are in equilibrium. Finally, amiloride inhibits $Na^+$-induced $H^+$ efflux, although one cannot tell from this experiment whether amiloride has penetrated the "ghost" lumen or is just acting on their outer surface.

### *4.2.8 $Na^+/Na^+$ Exchange*

""Ghosts" that contain $Na^+$ exchange this for $^{22}Na^+$ in the medium; this is probably a function of the $Na^+/H^+$ antiporter, already shown to be reversible. This is demonstrated in Fig. 4.11. "Ghosts" containing approx. 4 mM $Na^+$ were incubated in a medium containing 5mM $^{22}Na^+$, which is accumulated over about 30 min. A similar incubation was performed in the presence of the same concentration of non-radioactive $Na^+$. At 30 min, when, according to the first result, $^{22}Na^+$ uptake

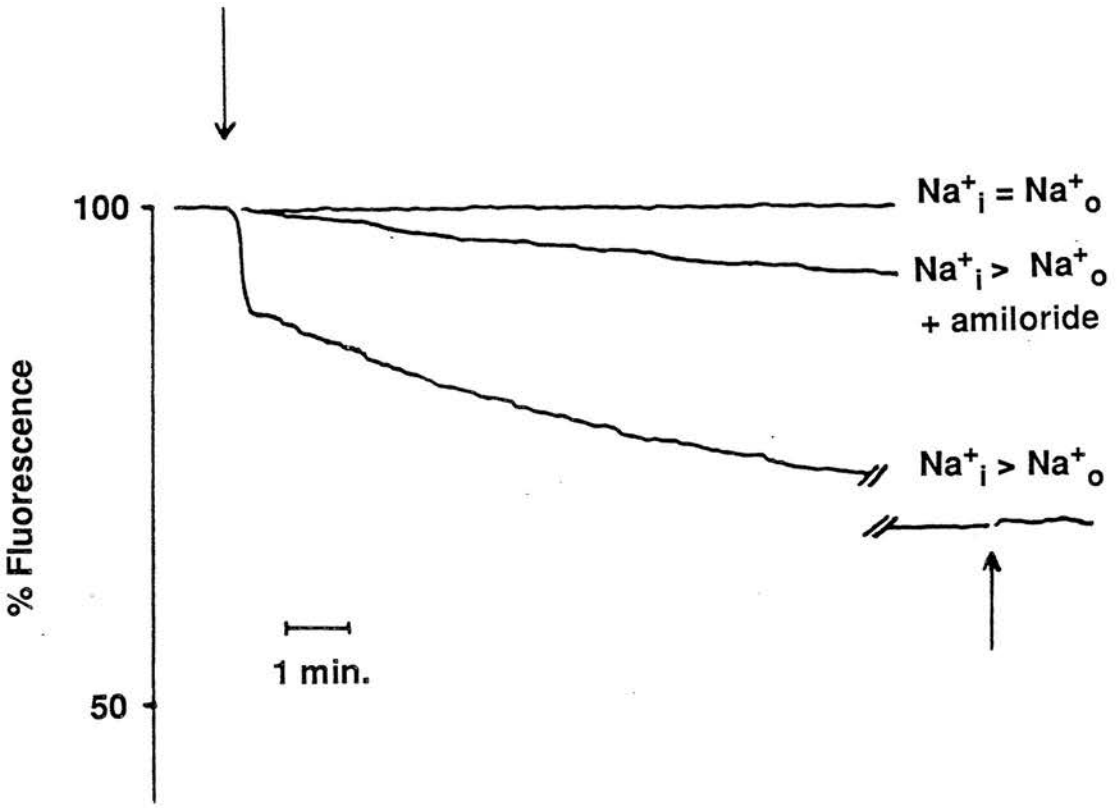



***Fig. 4.10 Acidification of $Na^+$-loaded "ghosts"***

"Ghosts" loaded with 25mM $Na_2SO_4$ at pH 7.0 were diluted into a medium (pH 7.0, 25°C) containing ACMA at the time shown by the first arrow to give a concentration of 0.12mg.ml$^{-1}$, and fluorescence was monitored. In the control experiment, the medium contained 25mM $Na_2SO_4$ so that no $Na^+$ gradient was established. In the other experiments the final medium contained 0.85mM $Na_2SO_4$ ($Na^+_o$ = 1.7mM), with or without 1mM amiloride. At the second arrow (30 min. after the start), either 10μM monensin or 10μM FCCP was added.

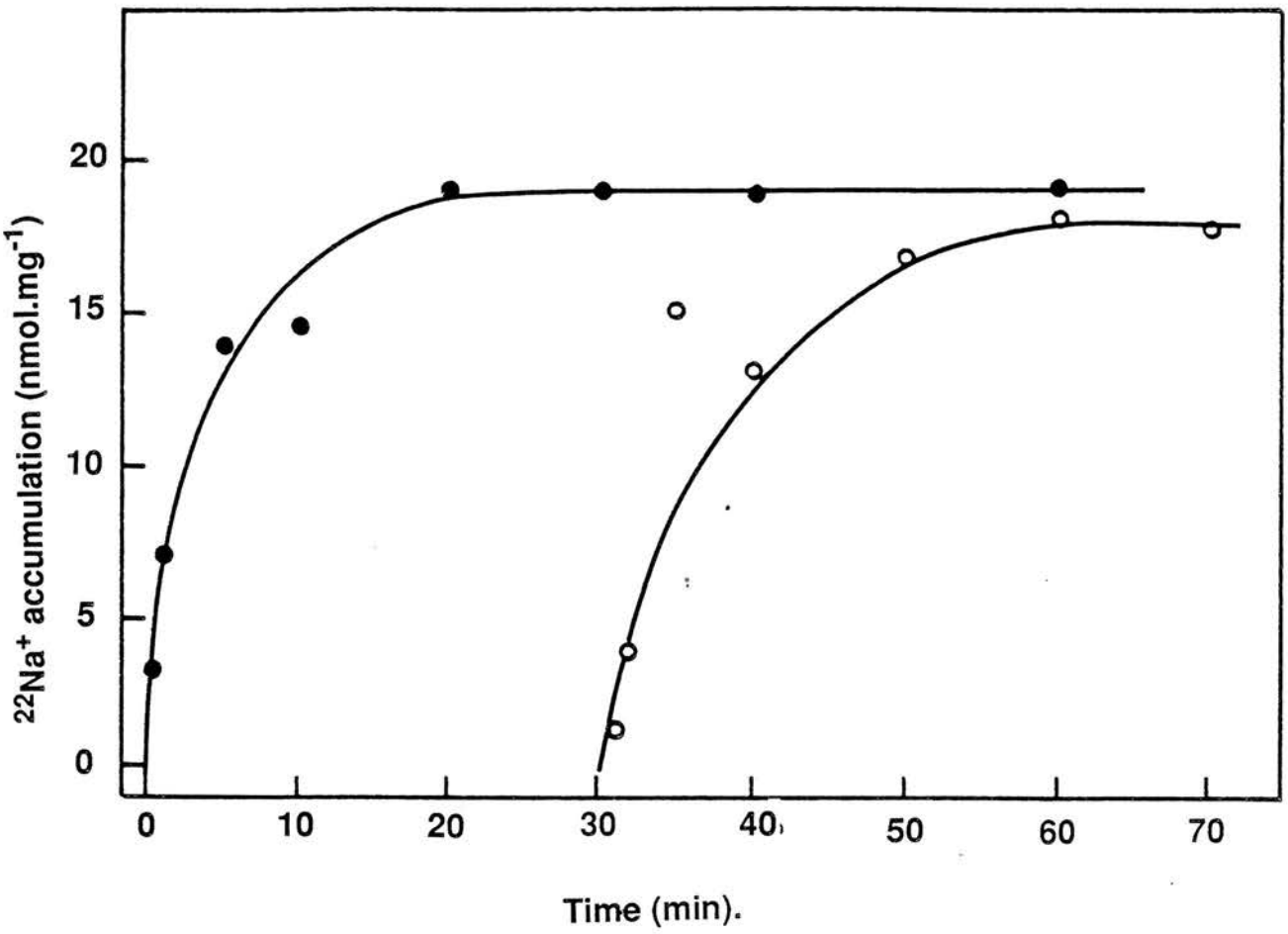



***Fig. 4.11 $Na^+/Na^+$ exchange by "ghosts"***

"Ghosts" containing 4mM $Na^+$ were incubated at 37°C in a medium with a similar concentration of $Na^+$ (5mM) at a protein concentration of 0.12mg.ml$^{-1}$. The medium was supplemented with $^{22}NaCl$ (1.5μCi.ml$^{-1}$) either at the start of the incubation (●) or after 30 min (○), and $^{22}Na^+$ accumulation was followed by filtration of 100μl aliquots.

had essentially ceased, a trace amount of $^{22}Na^{+}$ was added to the second incubation. Uptake of radioactivity occurred as in the first incubation, showing that at 30 min there is an equilibrium, with the rate of loss of $Na^{+}$ from the " ghosts" equalling the rate of uptake, the $^{22}Na^{+}$ having equilibrated across the membrane. The plateau value reached in Fig. 4.11 (18-19 $nmol.mg^{-1}$) is equivalent to an internal concentration of 5mM, if the internal volume of the "ghosts" is $3.6\mu l.mg^{-1}$ (Phillips & Allison, 1978).

### *4.3 Discussion*

The results presented in this Chapter provide clear evidence for the existence of a $Na^+/H^+$ antiporter in the chromaffin granule membrane. A variety of techniques has been employed to assay the exchange activity: measurement of $^{22}Na^+$ uptake in response to an imposed pH gradient or by ATP hydrolysis; generation of a pH gradient in response to an imposed $Na^+$ gradient; dissipation of a pH gradient resulting from $Na^+$ uptake; and $Na^+/Na^+$ exchange. Using ACMA quenching, the apparent $K_m$ for extravesicular $Na^+$ at pH 7.0 is 4.7mM; however, using a direct assay of $^{22}Na^+$ uptake in pH-jump experiments in the absence of ATP a value closer to 20mM was found, with a $V_{max}$ of the order of 150 to 200 $nmol.min^{-1}mg^{-1}$. This is similar to the $V_{max}$ of the $H^+$-ATPase of the granule membrane (Johnson *et al.*, 1982a; Percy *et al.*, 1985).

As with the $Na^+/H^+$ exchanger that is present in plasma membranes, the exchanger in the granule membrane appears to be electroneutral with a stoichiometry of 1:1, and is reversible. Inspection of Figs. 4.6 and 4.10 show that an apparent 30-fold $Na^+$ gradient ($Na^+_{in} > Na^+_{out}$) imposed across the membrane, supports the formation of 30-fold $H^+$ ($H^+_{in} > H^+_{out}$) gradient (i.e. $\Delta pH$ = 1.5 units), suggesting that the following equation is satisfied:

$$[Na^+]_i / [Na^+]_o = [H^+]_i / [H^+]_o \qquad (2)$$

At equilibrium, addition of monensin or FCCP (Fig. 4.10) does not disturb the trace. An attempt was made to measure the size of the $Na^+$ gradient at equilibrium using $^{22}Na^+$-loaded "ghosts" diluted into a $Na^+$ free medium, and monitoring the loss of $^{22}Na^+$ from the "ghost" lumen. Although the errors are large, a rough measurement showed that only a small amount of $Na^+$ is lost from the "ghosts"; therefore, the magnitude of the $Na^+$ gradients immediately following dilution and at equilibrium

seem to be much the same (results not shown).

In contrast with $Na^+$-induced $H^+$ uptake, $Na^+$ uptake by the "ghosts" in response to a pH-jump (Flg. 4.4) produced plateau values of $Na^+$ that were well below those predicted by eqn. (2). For example, in Fig. 4.4a a pH-jump of 2 pH units was imposed (i.e. a 100-fold gradient of $H^+$, inside over outside) although the $Na^+$ gradient at equilibrium was only 2.7 fold (inside over outside), assuming an internal volume for the "ghosts" of $3.6\mu l.mg^{-1}$ (Phillips & Allison, 1978). As Fig. 4.4c shows, there is a rapid dissipation of the transmembrane pH gradient and consequently a reduction in the driving force for $Na^+$ uptake.

Is the equilibrium condition above (eqn. 2) met *in vivo* ? If the pH gradient across the chromaffin granule membrane in intact cells is about 1.5 pH units (i.e. matrix pH = 5.5; cytosolic pH = 7.0), a 30 fold-gradient of $Na^+$ (matrix minus cytosol) could be sustained. The cytosolic concentration of $Na^+$ has not been measured, but is presumed to be between 1 and 5mM. Krieger & Gratzl (1982) have measured intragranular $Na^+$ to be 47mM, although this value depends on the value of the internal volume of the "ghosts", and whether the activity coefficient for $Na^+$ in the matrix is less than unity (the activity coefficients for $Na^+$, $K^+$ and $Ca^{2+}$ have been measured in isolated chromaffin granules and these experiments are described in Chapter 6). Therefore these calculations, though approximate, are consistent with the idea that the gradients are in equilibrium.

Recently, Ornberg *et al.*, (1988) have reported and electron probe X-ray microanalysis of cultured bovine chromaffin cells. They suggest that the high $Na^+$ content measured by Krieger-Brauer & Gratzl (1982) is an artifact arising from exchange of intragranular $K^+$ for $Na^+$ *post-mortem*, and during isolation of granules, and that the matrix is free of $Na^+$ *in situ*.. Although this seems unlikely from the results described (i.e. the presence of the relatively active $Na^+/H^+$ exchange activity in the membrane), it was clear that I never found the concentrations of $Na^+$ in the "ghosts" at equilibrium (Figs. 4.2 and 4.4) that would have been predicted from

eqn. 2. It is possible that the exchanger is not very active under physiological conditions (i.e., when the matrix pH is about 6.0) An additional possibility is that there is a third, as yet unidentified route of $Na^+$ transport across the granule membrane, complicating the analysis of the exchanges taking place here.

In common with other $Na^+/H^+$ antiporters, exchange is inhibited competitively by the diuretic drug amiloride; at physiological $Na^+$ concentrations, millimolar concentrations of amiloride are required to block the exchanger. The $K_i$ value found (0.26mM) is similar to that found in other systems, for example the $Na^+/H^+$ antiporter of the plasma membrane of chick cardiac cells (Lazdunski *et al.*, 1985) and cardiac sarcolemmal vesicles (Sieler *et al.*, 1985). Amiloride inhibits both $Na^+$ and $H^+$ fluxes into or out of the "ghost" lumen (e.g. Table 4.1 and Figs. 4.4, 4.8, 4.9 and 4.10) but does not effect rates of 5HT transport driven by Mg-ATP hydrolysis (Table 4.1), suggesting that the drug is indeed acting on the $Na^+/H^+$ exchanger.

The competitive behaviour of amiloride resembles that observed between tetrodotoxin and $Na^+$ for the voltage-gated $Na^+$ channel (Ritchie & Rogart, 1977; Lombert *et al.*, 1981). Tetrodotoxin and amiloride both have a guanidinium group that is essential for their inhibitory activity (see amiloride structure in the Appendix). However, in many systems, amiloride is not potent or specific enough for a thorough study of the physiological and molecular properties of the $Na^+/H^+$ antiporter. The competitive nature of its inhibition means that fairly high concentrations (millimolar) are required for inhibition under physiological conditions. High concentrations of amiloride also inhibit other cation translocating systems, such as the $Ca^{2+}/Na^+$ exchanger of rat brain (Schellenberg *et al.*, 1983) and kidney $(Na^+,K^+)$-ATPase (Soltoff & Mandel, 1983). The most active analogue of amiloride is ethylisopropylamiloride (EIPA), in which the 5-amino group of amiloride is substituted (see Appendix). EIPA is about 200 times more potent than amiloride: in chick cardiac cells at a physiological $Na^+$ concentration (140mM), the $K_i$ for inhibition of the antiporter by EIPA is 0.15μM (Vigne *et al.*, 1983). EIPA is without

effect on $Ca^{2+}/Na^{+}$ exchange of papillary muscle membranes (Siegl *et al.*, 1984) and $(Na^{+},K^{+})$- ATPase activities of A431 cells (Zhuang *et al.*, 1984). Therefore, EIPA can be considered to be a specific and high affinity inhibitor of the $Na^{+}/H^{+}$ antiporter, and does not interfere with other membrane cation transport systems in a variety of different cell types.

Is the $Na^{+}/H^{+}$ antiporter of the chromaffin granule identical to the plasma membrane $Na^{+}/H^{+}$ exchanger that has been identified in many cell types? (reviewed by Grinstein & Rothstein, 1986). The specificity and the $K_m$ values of the carriers appear similar, as are the $K_i$ values for amiloride (Lazdunski *et al.*, 1985; Seiler *et al.*, 1985). The main difference appears to be the lack of inhibition of the granule exchanger by EIPA, a rather surprising finding in view of its highly potent action on $Na^{+}/H^{+}$ antiporters in plasma membranes. This clearly merits further investigation.

# *Chapter Five*

# *$Ca^{2+}$ Transport by Chromaffin Granule "Ghosts"*

## *5.1 Introduction*

Isolated plasma membrane vesicles have proved to be a particularly useful model system for studies of several membrane transport processes. These vesicles are largely devoid of the cytoplasmic constituents of the intact cell, and their metabolic activities are determined by the presence of the enzymes in the membrane itself. For example, the energy source for transport of a particular substrate can be determined by studying which substances stimulate solute accumulation.

In electrically excitable cells such as those from heart and skeletal muscle, $Ca^{2+}/Na^{+}$ exchange plays a key role in $Ca^{2+}$ homeostasis. $Ca^{2+}$ is transported across the plasma membrane coupled to $Na^{+}$ movement in the opposite direction and $Ca^{2+}$ uptake depends on the size of the $Na^{+}$ concentration gradient and on the transmembrane electrical potential. Early studies of $Ca^{2+}/Na^{+}$ exchange in cardiac tissue, for example, were based on isotopic flux data using intact muscle preparations (Jundt *et al.,* 1975; Wendt & Langer, 1977). However, cells possess multiple $Ca^{2+}$ transport systems, and unequivocal assignment of $Na^{+}$ or $Ca^{2+}$ flux to $Ca^{2+}/Na^{+}$ exchange was therefore difficult. In the heart, much new information has been obtained using $Na^{+}$-loaded plasma membrane vesicles derived from sarcolemma, first described by Reeves & Sutko (1979). Briefly, $Na^{+}$-loaded vesicles are diluted into a $Na^{+}$-free medium containing $^{45}Ca^{2+}$, and the $^{45}Ca^{2+}$ accumulation in exchange for intravesicular $Na^{+}$ is assessed by rapid filtration of vesicles onto nitrocellulose filters, followed by liquid scintillation counting. Using this technique, the kinetics and stoichiometry of $Ca^{2+}/Na^{+}$exchange have been extensively characterised (Reeves & Sutko, 1983; Philipson, 1985). In addition, $Ca^{2+}/Na^{+}$ exchange has been measured using spectroscopic (eg. Arsenazo III; Caroni and Carafoli, 1983) or ion-specific electrode (Caroni *et al.,* 1980) techniques.

In view of the difficulties noted in Chapter 3 for measuring $Ca^{2+}/Na^{+}$ exchange activities across the membranes of intact chromaffin granules, I have used resealed membrane vesicles ("ghosts") to extend the studies of Phillips (1981) and

Krieger-Brauer & Gratzl (1983). "Ghosts" are easily prepared (Apps *et al.,* 1980) by lysing freshly isolated chromaffin granules in hypo-osmotic solutions, which releases the majority of their matrix constituents. After restoration of the osmolarity, the resealed membranes may be collected by centrifugation and purified from mitochondrial contamination on a density gradient. "Ghosts" have proved excellent for studies of chromaffin granule membrane transport as their internal environment can be manipulated by enclosing solutes of almost any required composition during resealing.

In this chapter, I have investigated $Ca^{2+}/Na^{+}$ exchange using $Na^{+}$-loaded "ghosts" with methods similar to those described by Reeves & Sutko (1979) and others, for $Na^{+}$-loaded sarcolemmal vesicles. In addition, I show that $Ca^{2+}$ uptake by "ghosts" is not directly coupled to ATP-dependent $H^{+}$-translocation across the granule membrane, but is dependent on the uptake of $Na^{+}$ into the "ghosts" lumen catalysed by the $Na^{+}/H^{+}$ antiporter described in the previous chapter.

## 5.2 Results

### 5.2.1 Calibration of the $Ca^{2+}$ Electrode

The Nernstian behaviour of the $Ca^{2+}$-sensitive minielectrode, constructed as described in Chapter 2, was determined using buffered sucrose solutions at 37°C containing 0.1 to 10mM $Ca^{2+}$ but lacking EGTA or HEDTA. Because $Ca^{2+}$ is divalent, the $Ca^{2+}$ electrode gives only half the slope of a normal $H^+$ electrode (theoretical value 30.5mV/pCa instead of 61mV/pCa at 37°C). A value of 29.5 mV/pCa unit was found.

A series of $Ca^{2+}$-EGTA and $Ca^{2+}$-EDTA buffers was then used to check the slope and sensitivity of the electrode (Bers, 1982). Note that nominal chelator concentrations of 1.0mM EGTA and 0.2mM HEDTA were used (see Chapter 2). With each solution, the potential recorded by the electrode ($V_{Ca}$, mV) was measured; the free [$Ca^{2+}$] predicted for those solutions was calculated, assuming that the electrode response continued to be 29.5 mV/pCa unit, and using the association constants for EGTA and HEDTA given by Sillen and Martell (1971). These association constants were not measured under the precise ionic conditions of the present measurements. From these free $Ca^{2+}$ concentrations and the known concentrations of the chelating agents, the bound $Ca^{2+}$ concentration at each point was calculated. Each value was then plotted in a Scatchard plot (Fig. 5.1).

From Fig. 5.1 it can be seen that the electrode behaves ideally with both types of $Ca^{2+}$ buffer over the whole range of free $Ca^{2+}$ used. The plots do not bend back towards the origin as would be expected if the electrode failed to indicate true free $Ca^{2+}$ at very low levels. On these plots, the intercept on the x-axis is equal to the total [EGTA] or total [HEDTA], and values of 0.95mM and 0.19mM were found respectively. These are slightly lower than would be expected from the percent nominal total [EGTA] or [HEDTA] quoted by Sigma for these reagents (97% pure for EGTA, 98% pure for HEDTA). The apparent $Ca^{2+}$-EGTA or $Ca^{2+}$-HEDTA association

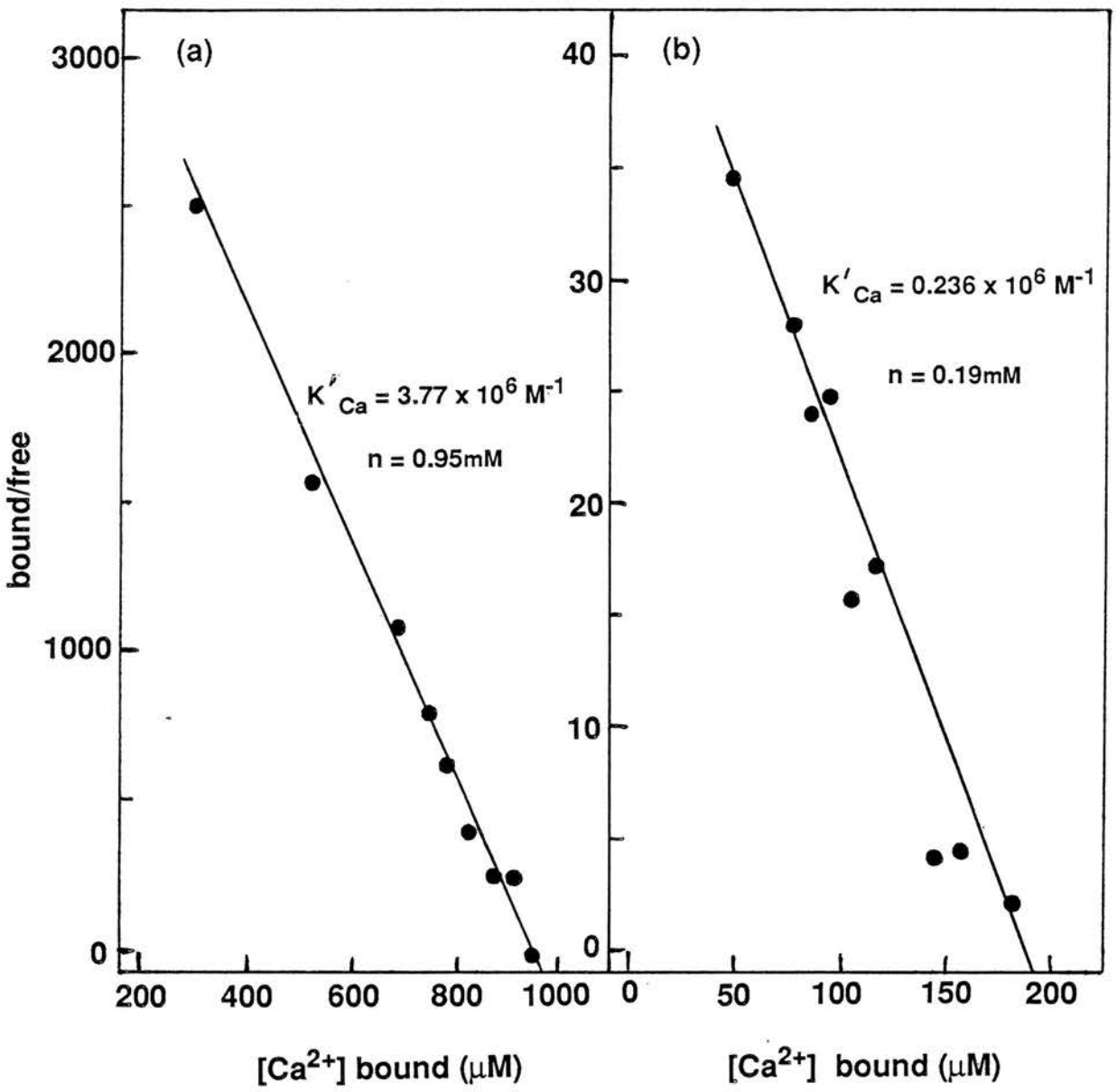



***Fig. 5.1 Scatchard plots for the determination of $K'_{Ca}$, total [EGTA] and total [HEDTA]***

The plots are derived from sets of solutions used to calibrate a $Ca^{2+}$-minielectrode. These contained either (a) EGTA, or (b) HEDTA as chelating agent. For details see the text. The lines are "best-fit" by linear regression analysis.

constant ($K'_{Ca}$) is given by the slope of the Scatchard plot. For EGTA this was found to be 3.77 x $10^6$ $M^{-1}$ (Fig. 5.1a), a value that can be compared to that found by Bers (1982) for EGTA in a medium of ionic strength 110mM at 37°C using a different electrode system (3.06 $\pm$ 0.06 x $10^6$ $M^{-1}$ ). The $K'_{Ca}$ for $Ca^{2+}$-HEDTA solutions from Fig. 5.1b was found to be 0.236 x $10^6$ $M^{-1}$ (not measured by Bers, 1982).

Finally, the pCa (-log free [$Ca^{2+}$]) was recalculated from the total [$Ca^{2+}$] using the values of $K'_{Ca}$ and the chelator concentration (n, in mM) determined above (i.e. from Fig. 5.1). The calculation was performed using the $\alpha$-coefficient method of Schwarzenbach (1957) as described in Perrin & Dempsey (1974). This method is very convenient for calculating free metal ion concentrations when excess chelating agent is present. As shown by the resultant electrode calibration curves (Fig. 5.2) the electrode is linear down to a free [$Ca^{2+}$] of 0.4$\mu$M (pCa 6.4) and is therefore of sufficient sensitivity for experiments involving the use of physiological concentrations of free $Ca^{2+}$.

For comparison, Table 5.1 shows values for solutions containing various free $Ca^{2+}$ concentrations within the physiological range, with EGTA or HEDTA as the buffer, calculated using either the algorithm of Storer & Cornish-Bowden (1976) and the dissociation constants for EGTA and HEDTA tabulated by Sillen and Martell (1971), or by the $\alpha$-coefficient method (Perrin & Dempsey, 1974) using the values of $K'_{Ca}$ and the chelator concentration (n) determined in the present work (Fig. 5.1).

As can be seen from Table 5.1, the two methods give slightly different values for free [$Ca^{2+}$]. In the work presented in this thesis, I have used the constants obtained from Fig. 5.1, since these were measured under conditions closely similar to those employed in experiments with membrane vesicles (buffered sucrose at low ionic strength).

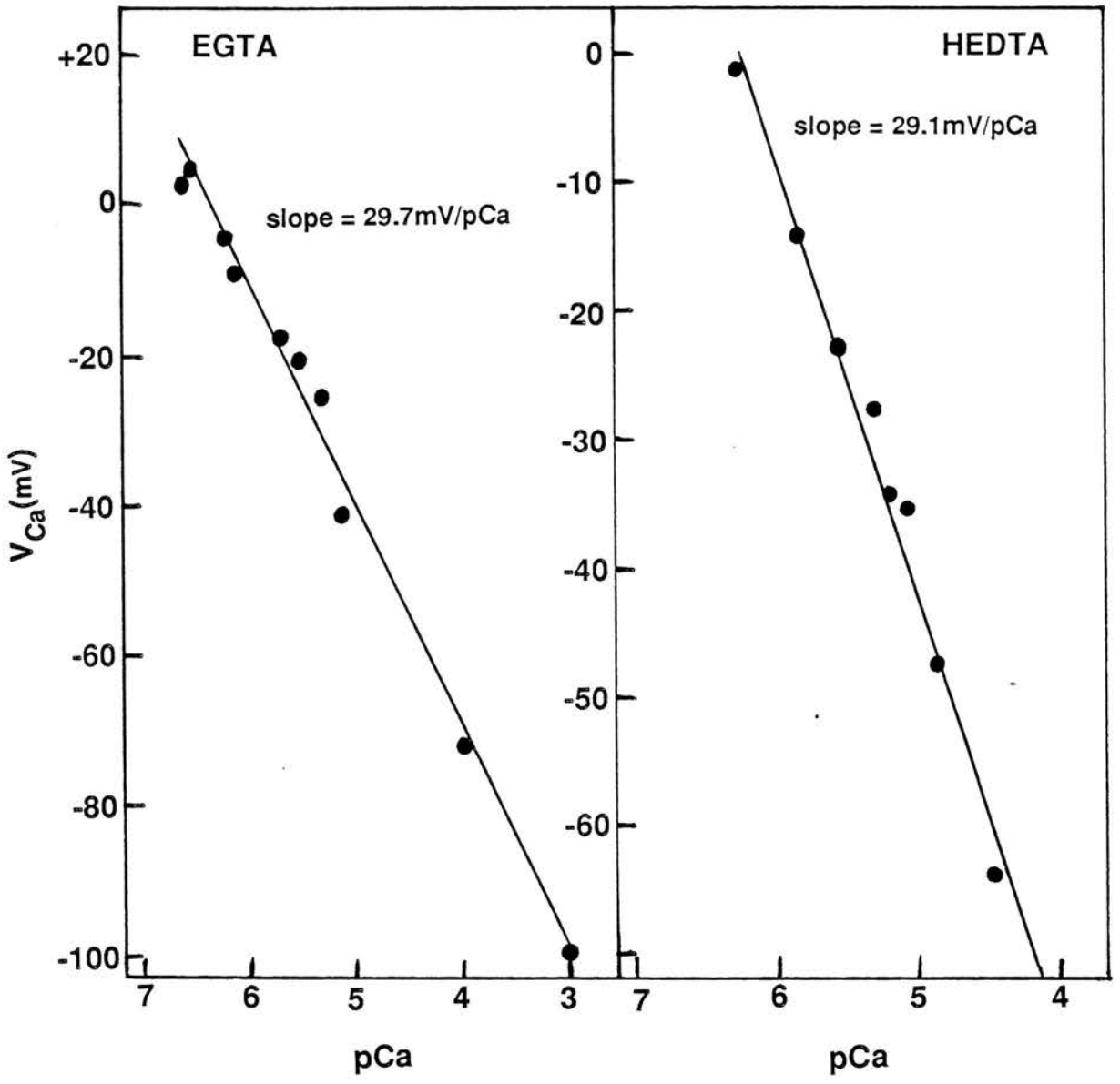



*Fig. 5.2 Response of the $Ca^{2+}$ electrode*

Plots of $V_{Ca}$ against pCa calculated from total [$Ca^{2+}$], $K'_{Ca}$ and chelator concentration (n) determined from the Scatchard plots shown in Fig. 5.1.

*TABLE 5.1 The use of EGTA and HEDTA as $Ca^{2+}$ buffers.*

| | EGTA | | | HEDTA | |
|---|---|---|---|---|---|
| Total $Ca^{2+}$ (mM) | Free $Ca^{2+}$ ($\mu$M) $\alpha$-coefficient method, using constants from Fig. 5.1a | Free $Ca^{2+}$ ($\mu$M) Storer-Cornish Bowden method using constants from Sillen & Martell(1971) | Total $Ca^{2+}$ (mM) | Free $Ca^{2+}$ ($\mu$M) $\alpha$-coefficient method, using constants from Fig. 5.1b | Free $Ca^{2+}$ ($\mu$M) Storer-Cornish Bowden method, using constants from Sillen & Martell(1971). |
| 0.045 | 0.08 | 0.064 | 0.03 | 0.79 | 0.70 |
| 0.09 | 0.24 | 0.187 | 0.05 | 1.52 | 1.53 |
| 0.15 | 1.00 | 0.78 | 0.07 | 2.48 | 2.14 |
| 0.175 | 3.10 | 2.27 | 0.10 | 4.73 | 3.94 |
| 0.18 | 4.78 | 3.19 | 0.115 | 6.54 | 5.25 |
| 0.183 | 6.94 | 4.00 | 0.13 | 9.22 | 7.00 |

**All solutions contained 0.19mM EGTA or HEDTA and the total concentrations of $Ca^{2+}$ listed, in 0.3M sucrose, 10mM Hepes-KOH, pH 7.0 at 37°C.**

### *5.2.2 $Na^+_i$-dependent $Ca^{2+}$ Uptake by "Ghosts"*

All $Ca^{2+}/Na^+$ exchange measurements have been made by diluting $Na^+$-loaded chromaffin granule "ghosts" into a $Na^+$-free medium containing $^{45}Ca^{2+}$. The free $Ca^{2+}$ concentrations in dilution media have been calculated for the concentrations of total [EGTA] or total [HEDTA] shown in Table 5.1.

In the experiment shown in Fig. 5.3 $Na^+$-loaded "ghosts" (containing 50mM $Na^+$ and 0.1mM EGTA) were diluted 25-fold into nominally $Na^+$-free media containing either 50mM $K^+$, thus generating a large outwardly directed $Na^+$ gradient ($Na^+_{in} > Na^+_{out}$), or into 50mM $Na^+$ ( in which case no $Na^+$ gradient is generated). No ATP was present in the external media. With a $Na^+$ gradient, $^{45}Ca^{2+}$ is accumulated rapidly and reaches a plateau value of 20 $nmol.mg^{-1}$ after about 40 min. The plateau value corresponds to a nominal internal $Ca^+$ concentration of about 6.6mM, taking into account the internal volume of the "ghosts" ($3\mu l.mg^{-1}$ protein) and their internal EGTA concentration (0.1mM). In contrast, in the absence of a $Na^+$ gradient, only one-tenth as much $Ca^{2+}$ is accumulated.

The cation specificity of exchange was examined by diluting $K^+$-loaded "ghosts" (containing 50mM $K^+$ and 0.1mM EGTA) into a $Na^+$-free medium plus $^{45}Ca^{2+}$. Very little $^{45}Ca$ uptake was apparent, with values lower than those obtained in the control experiment in the absence of a $Na^+$ gradient in Fig. 5.3.

Thus, $Ca^{2+}/Na^+$ exchange is being directly demonstrated across the vesicle membrane, the 25-fold $Na^+$ gradient ($[Na^+]_i$ = 50mM, $[Na^+]_o$ = 2mM) providing the only readily apparent driving force for $Ca^{2+}$ uptake by the $Na^+$-loaded vesicles. At the plateau value in Fig. 5.3 there is an apparent $Ca^{2+}$ gradient across the membrane of about 330-fold ($[Ca^{2+}]_i$ = 6.6mM, $[Ca^{2+}]_o$ = 20$\mu$M).

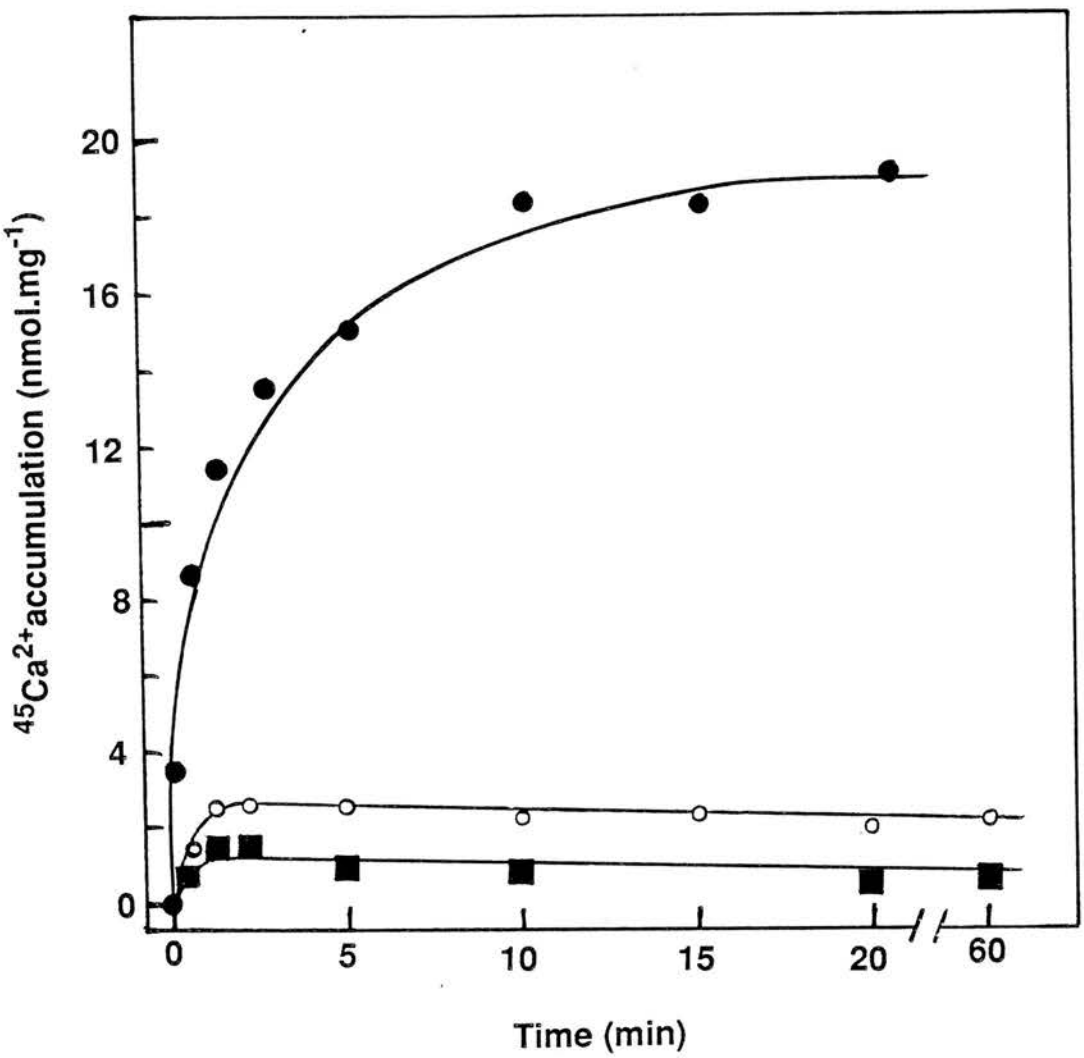



*Fig. 5.3 $Na^+_i$-dependent $Ca^{2+}$ uptake by "ghosts"*

$Na^+$-loaded "ghosts" (containing 50mM $Na^+$, pH 7.0) were added to a media (37°C, pH 7.0) containing either 50mM $Na^+$ (○) or 50mM $K^+$ (●), and $^{45}Ca^{2+}$ uptake was measured after filtration of 100µl aliquots. $K^+$-loaded "ghosts" were diluted into a $K^+$-free medium (■), and $Ca^{2+}$ uptake was monitored.

### *5.2.3 Kinetics of $Ca^{2+}/Na^{+}$ Exchange*

This preparation should allow fairly accurate determination of the $K_m$ and $V_{max}$ for $Ca^{2+}$ during $Ca^{2+}/Na^{+}$ exchange ($Na^{+}_i$-dependent $Ca^{2+}$ uptake), provided accurate initial rates of $Ca^{2+}$ uptake could be measured. This was achieved by diluting $Na^{+}$-loaded "ghosts" into $Na^{+}$-free media containing micromolar concentrations of free $Ca^{2+}$, buffered using EGTA or HEDTA, and quenching the subsequent $Ca^{2+}$ uptake after 4 sec. The rates of uptake were rather variable between different "ghosts" preparations, but at 37°C linearity was always maintained for at least 5 sec and usually up to 8 sec. Transport in two different vesicle preparations displayed $K_m$ values for $Ca^{2+}$ of 0.9μM and 1.0μM, and $V_{max}$ values of 5.5 and 8.1nmol.$mg^{-1}$. 4 $sec^{-1}$, with $Ca^{2+}$ buffered with EGTA. The kinetics were slightly different when HEDTA was used: $K_m$ values of 2.5μM and 3μM, and $V_{max}$ values of 2.9 and 4.0 nmol.$mg^{-1}$.4 $sec^{-1}$, were found. The mean $K_m$ and $V_{max}$ values obtained with the two membrane preparations are shown in the Hanes plots in Fig. 5.4.

### *5.2.4 Relationship Between $Ca^{2+}$ and $Na^{+}$ Transport and the $H^{+}$-translocating ATPase*

In Chapter 4 evidence was presented for the presence of an amiloride-sensitive $Na^{+}/H^{+}$ antiporter in the chromaffin granule membrane. This carrier can catalyse $Na^{+}$ uptake via the antiporter by utilising the $H^{+}$ gradient that is generated across the granule membrane by the $H^{+}$-translocating ATPase (acid inside). However, $Ca^{2+}$ uptake by $Ca^{2+}/Na^{+}$ antiport is presumably not directly coupled to the transmembrane $H^{+}$ gradient; therefore, it would be desirable to show that $Ca^{2+}$ uptake can be driven by internal $Na^{+}$, the latter being accumulated by exchange with internal protons that have entered the granule matrix as a result of ATP hydrolysis by the ATPase.

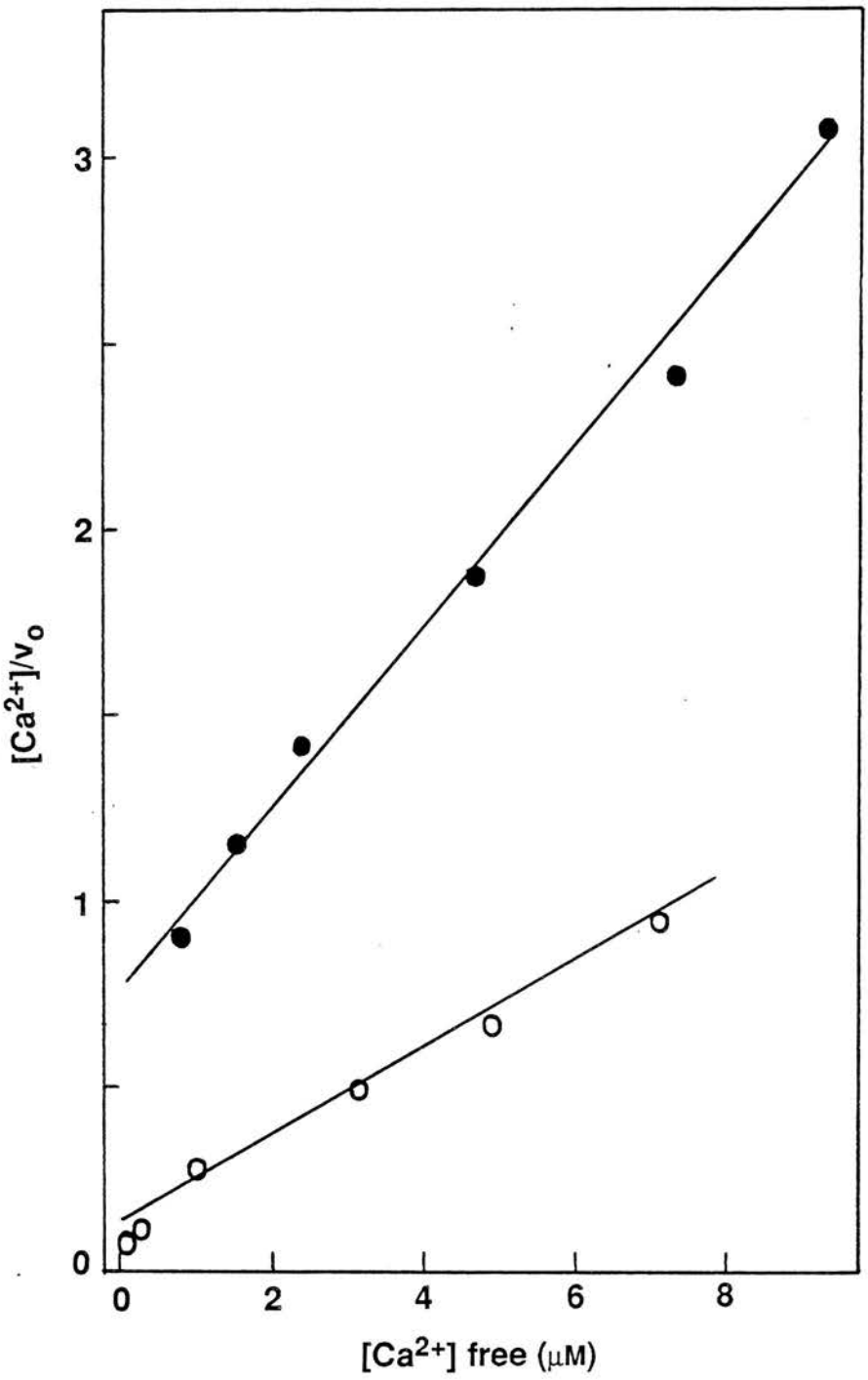



***Fig. 5.4 Kinetics of $Ca^{2+}/Na^{+}$ exchange by "ghosts"***

$Na^{+}$-loaded "ghosts" (0.11mg.$ml^{-1}$) were diluted into media (pH 7.0, 25°C) with various extravesicular $Ca^{2+}$ concentrations, buffered with either **(a)** EGTA (○) or **(b)** HEDTA (●). $^{45}Ca^{2+}$ incorporation was assessed by terminating $Ca^{2+}$ uptake after 4 sec., and initial rates ($v_o$) are in nmol.$mg^{-1}$.4$sec^{-1}$.

In the experiments described below, "ghosts" resealed in 10mM EDTA-TMA, pH 7.0, and nominally free of internal $Ca^{2+}$ and $Na^{+}$, were used. Incubation of "ghosts" in a medium containing buffered 0.3M sucrose, MgATP and 18μM free $^{45}Ca^{2+}$ leads to an incorporation of $^{45}Ca^{2+}$ (Fig. 5.5). Uptake is relatively slow, and after 30 min 5.2 ± 0.52 nmol $Ca^{2+}.mg^{-1}$ have been accumulated (mean ± S.E.M. for 4 independent determinations). Continued incubation leads to a further slow uptake: after 3 hrs incubation an internal $Ca^{2+}$ content of about 11nmol.$mg^{-1}$ protein has been reached. At all time points, about 90% of the accumulated $Ca^{2+}$ is released if the ghosts are subjected to hypo-osmotic shock, and no uptake occurs at 0°C. Inclusion of approx. 1mM free $Mg^{2+}$ in the incubation medium inhibits the uptake by about 75% (results not shown)

If the uncoupler FCCP is present in the incubation medium as well as MgATP, uptake of $Ca^{2+}$ is unaffected, and values are superimposable with those over the first 30 min. That the uptake of $Ca^{2+}$ is independent of energisation by the ATPase is also shown by the fact that identical uptake is found in the absence of ATP, using 18μM $Ca^{2+}$ in the presence of EGTA. The free $Ca^{2+}$ concentration within the matrix at any point will be determined by the total internal $Ca^{2+}$ concentration (which itself is estimated from the internal volume of the "ghosts"), the internal EDTA concentration and the internal pH. This is discussed in more detail below.

If $Na^{+}$ (5mM) is added to the medium after 30 min., there is a stimulation of the rate and extent of $Ca^{2+}$ accumulation, over and above the existing "slow" rate of $Ca^{2+}$ uptake. After a further 60 min the internal $^{45}Ca^{2+}$ content has reached a value of 12 nmol.$mg^{-1}$ protein, compared with 7.5 nmol.$mg^{-1}$ at the same time in the absence of added $Na^{+}$ (Fig. 5.5). In experiments with EGTA present, but no ATP, a transmembrane pH gradient is not generated, and the internal and external pH's remain the same (i.e. pH 7.2). Uptake of $^{45}Ca^{2+}$, monitored up to 60 min., was identical to that in the presence of MgATP (Fig. 5.5). Addition of $Na^{+}$ (5mM) at this point, caused, in some experiments, a slight efflux of $Ca^{2+}$, presumably due to exchange of external $Na^{+}$ with internal $Ca^{2+}$ (Phillips, 1981). However, $Ca^{2+}$

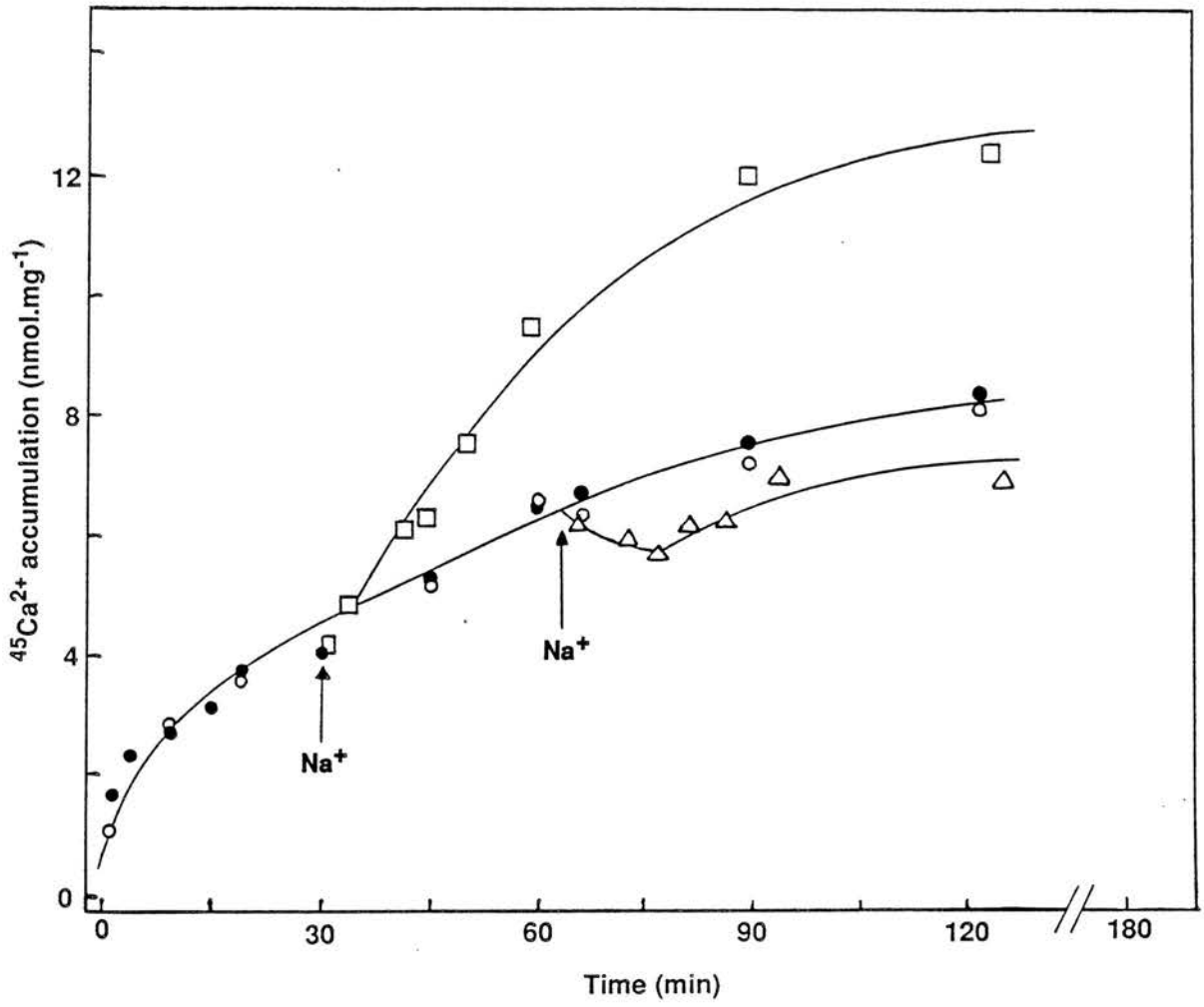



***Fig. 5.5 Uptake of $Ca^{2+}$ by chromaffin granule "ghosts"***

"Ghosts" (containing 10mM EDTA, pH 7.2) were incubated in a medium (30°C) containing 0.3M sucrose, 10mM Hepes-TMA, pH 7.2, 40mM TMA-iodide, and 18.4μM free $Ca^{2+}$ obtained using either MgATP (●) or EGTA (○). At the times shown, $Na^+$ (5mM) was added and the effect on the uptake of $Ca^{2+}$ with MgATP (□) or EGTA (△)was monitored; the effects of 10μM FCCP or 10μM bafilomycin were superimposable on the curves with MgATP or EGTA before (●,○) or after (△) the addition of $Na^+$.

uptake was not stimulated by $Na^+$, as it was in the presence of ATP, and a new equilibrium position was quickly reached which is maintained for at least a further 60 min. (Fig. 5.5). The chromaffin granule membrane $H^+$-ATPase produces a large acidification of the "ghost" matrix when a permeant anion, such as iodide is present in the incubation medium together with MgATP. For example, a pH gradient (inside acidic) of about 2 pH units (generated at the expense of a membrane potential) can be established across the ghost membrane using 40mM KI (Phillips & Allison, 1978). I have investigated the development of a pH gradient driven by MgATP hydrolysis, under the conditions described in Fig. 5.5. Ghosts were incubated in a medium containing MgATP, 18μM free $Ca^{2+}$ (but no $^{45}Ca^{2+}$) and 40mM TMA-iodide; the formation of a pH gradient was followed by including either a trace amount of [$^{14}$C]methylamine, and filtering samples as described in Chapter 2, or a small amount of ACMA and monitoring the uptake and quenching of its fluorescence. A typical result is shown in Fig. 5.6. A $\Delta$pH of about 1.0 unit is generated after 2 min, with an equilibrium value for $\Delta$pH of 1.9 units being reached after about 20 min. (Fig. 5.6a). This $\Delta$pH is constantly sustained by the hydrolysis of ATP by the "ghost" membrane's $H^+$-translocating ATPase.

Addition of 5mM $Na^+$ produces a slight fall in this $\Delta$pH, consistent with $H^+$ efflux in exchange for external $Na^+$. However, a new steady state is quickly attained and this $\Delta$pH (1.8 units) is maintained for a considerable time.

The effect of repeated additions of $Na^+$ on $H^+$-ATPase generated $\Delta$pH is more conveniently studied using a continuous recording of ACMA fluorescence (Fig. 5.6b). Again, an equilibrium $\Delta$pH of 1.9 pH units (inside minus outside) is achieved after 20 mins under these conditions (read from Fig. 4.7b, Chapter 4). Addition of $Na^+$ to 2mM results in a fall in $\Delta$pH which slightly "overshoots" before returning to a stable level after about 1 min. The net fall in $\Delta$pH is approx. 0.07 pH units. The total amount of $Na^+$ added (12mM) produces a net fall in $\Delta$pH of 0.4 units to a final value of 1.5 pH units ( a 30-fold concentration gradient, inside over outside).

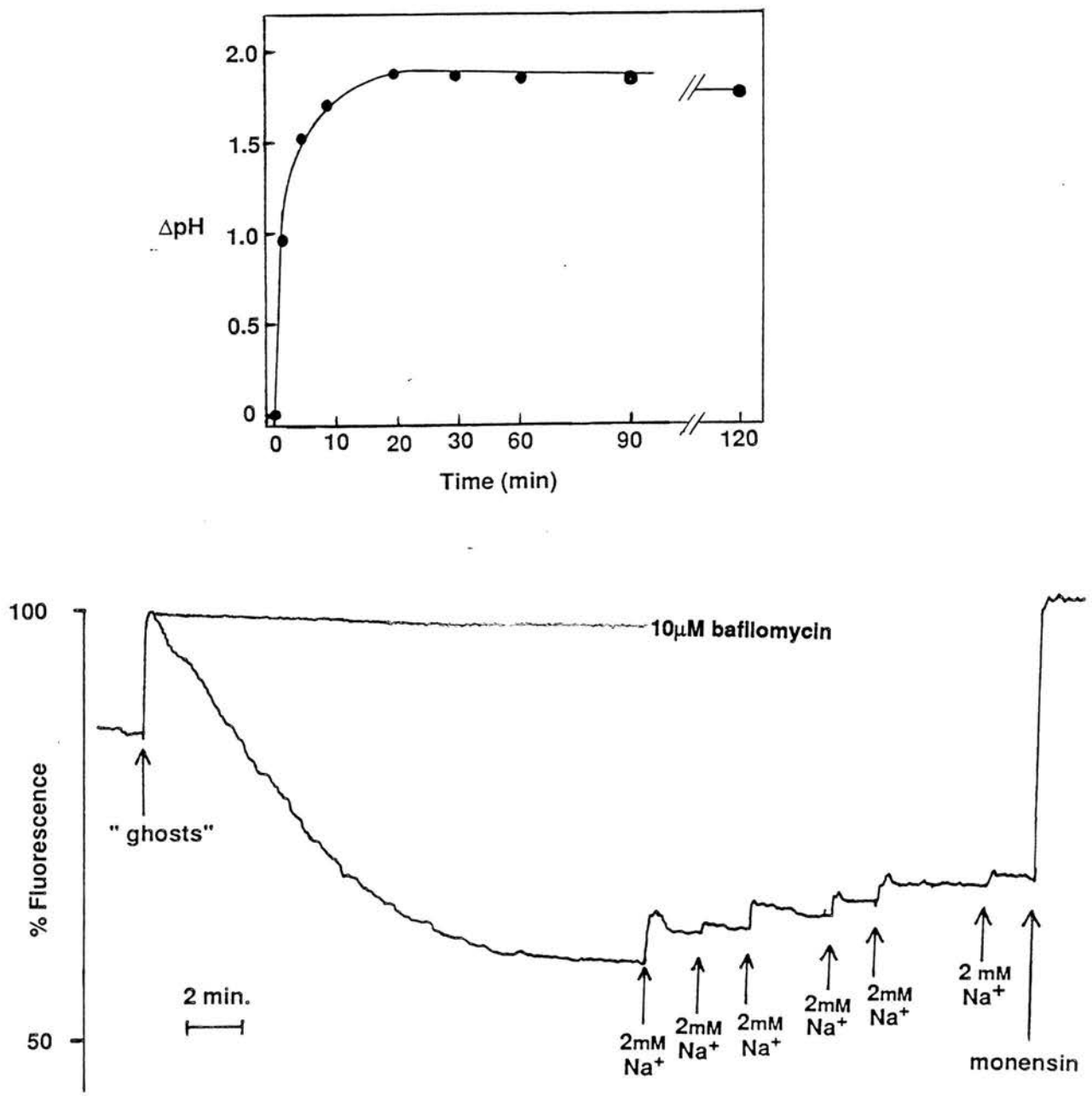



***Fig. 5.6 Generation of a pH gradient by "ghosts"***

"Ghosts" were incubated in the presence of MgATP, 18.4μM free $Ca^{2+}$ and 40mM TMA-iodide as shown in Fig. 5.5; "ghost" acidification was followed by monitoring either **(a)** the accumulation of [$^{14}$C]methylamine, or **(b)** the quenching of ACMA fluorescence. The effect of added $Na^+$ or the inclusion of 10μM bafilomycin on the pH gradient was followed. In **(b)**, ΔpH was read from the calibration graph (Fig. 4.7b) shown in Chapter 4.

I attempted to measure the accumulation of $Na^+$ by incubating EDTA-loaded "ghosts" as in Fig. 5.5. without any $^{45}Ca^{2+}$, and after 30 min adding 2.4mM $^{22}NaCl$. The $Na^+/H^+$ antiporter has a relatively high $K_m$ (about 5 mM, see Chapter 4) and therefore such an experiment is difficult because $^{22}Na^+$ is masked by non-radioactive $Na^+$ in the medium; of the $^{22}Na^+$ c.p.m. in the filtered "ghosts", 80% was accounted for by lysis-insensitive "background" c.p.m. remaining on the nitrocellulose filters. Very little $^{22}Na$ was accumulated in the ghost matrix (3.3 $nmol.mg^{-1}$ protein, equivalent to about 1.1mM internal $Na^+$, compared with 2.4mM externally). This makes a direct quantitative comparison between the $H^+$ gradient and $Na^+$ gradient impossible. Clearly some internal $Na^+$ is immediately lost by exchange with external $Ca^{2+}$, hence the increase in $Ca^{2+}$ uptake over and above the basal level when $Na^+$ is added to "ghosts" maintaining an acidic matrix generated by the $H^+$-translocating ATPase. Nevertheless, $Na^+$ does not come into equilibrium with the measured $H^+$ electrochemical gradient. An internal $Na^+$ concentration of about 150mM would be expected if the transmembrane $Na^+$ and $H^+$ concentration gradients come into equilibrium. This would be equivalent to an internal $Na^+$ content of 450 $nmol.mg^{-1}$ protein, if the internal volume of the "ghosts" is $3\mu l.mg^{-1}$, and would, therefore, have been easily measurable using $^{22}Na^+$ (see also Chapter 4).

I have also used the assays shown in Figs. 5.5 and 5.6 to demonstrate the effect on $Ca^{2+}$ transport of bafilomycin $A_1$, a macrolide antibiotic from *Streptomyces sp.* that is a highly potent and specific inhibitor of vacuolar $H^+$-ATPases, including the chromaffin granule $H^+$ pump (Bowman *et al.*, 1988).

In the presence of as little as 0.2μM bafilomycin, proton translocation and acidification of the ghost matrix is completely abolished, as shown in Fig. 5.6b. When bafilomycin (10μM) is included in the incubation medium together with MgATP and 18μM free $Ca^{2+}$ the usual slow rate of $Ca^{2+}$ uptake is observed (Fig. 5.5). However, subsequent addition of 5mM $Na^+$ does not promote further $Ca^{2+}$ accumulation, again demonstrating the requirement for a transmembrane proton gradient (acid inside) and external $Na^+$ for the $Ca^{2+}$ uptake response.

### *5.2.5 Free $Ca^{2+}$ Concentrations within the "Ghost" Matrix*

From the results shown in Figs. 5.5 and 5.6 the free $Ca^{2+}$ concentrations within the "ghosts" were calculated using the algorithm method described by Storer & Cornish-Bowden (1976), and these are presented in Table 5.2. Total matrix $Ca^{2+}$ concentrations (Total [$Ca^{2+}$], μM) was calculated assuming an internal volume for the "ghosts" of 3μl.mg$^{-1}$ protein, slightly lower than the value of 3.6μl.mg$^{-1}$ determined by Phillips & Allison (1978) at lower osmolarity. In all cases, ΔpH varied between 1.7 and 1.9 pH units (corresponding to methylamine concentration ratios of 50 and 80, respectively) and the external pH was 7.2, as measured with a pH electrode.

The internal EDTA concentration was assumed to be10mM, the concentration used in the granule lysis medium. In the presence of external MgATP, free $Ca^{2+}$ within the "ghost" matrix increased from 2.4 μM at 30 min. to 4.7 μM (mean of 90 and 120 min values) after addition of 5mM $Na^+$; in the absence of added $Na^+$, free $Ca^{2+}$ remained about the same (2.5 μM, mean of 90 and 120 min values). In both cases, the free $Ca^{2+}$ concentration after 120 mins would be higher if it were not for the 0.1 pH unit increases in matrix pH (from pH 5.4 to pH 5.5) that occurs between 90 and 120 mins (Table 5.2). This increase may be due, however, to "ghost" breakage during the prolonged incubation period. Nevertheless, the level of free $Ca^{2+}$ attained within the ghosts maintaining an acidic matrix, although low, is doubled after addition of $Na^+$ to the external medium.

When MgATP is absent from the incubation medium, or if it is present together with bafilomycin, proton translocation and matrix acidification does not occur. Nevertheless, total $Ca^{2+}$ uptake is not affected although the free matrix concentrations are extremely low (Table 5.2), about 5 x$10^3$-fold lower than their values in the presence of MgATP; this is due to the relatively high matrix pH (7.2) and consequent strong binding of $Ca^{2+}$ to EDTA. This suggests that, whatever the mechanism of this "passive" $Ca^{2+}$ entry, it is limited by some kinetic factor, rather than by the size of the transmembrane $Ca^{2+}$ gradient.

***TABLE 5.2 Free $Ca^{2+}$ concentrations in the "ghost" matrix.***

| | | +MgATP | | | -MgATP | |
|---|---|---|---|---|---|---|
| Time (min) | Matrix pH | Matrix Total $Ca^{2+}$ (mM) | Matrix Free $Ca^{2+}$ (μM) | Matrix pH | Matrix Total $Ca^{2+}$ (mM) | Matrix Free $Ca^{2+}$ (μM) |
| 10 | 5.45 | 0.83 | 0.70 | 7.2 | 0.83 | $0.23 \times 10^{-3}$ |
| 30 | 5.30 | 1.33 | 2.4 | 7.2 | 1.33 | $0.39 \times 10^{-3}$ |
| (5mM $Na^+$ added) | | | | | | |
| 60 | 5.38 | 2.83 | 3.4 | 7.2 | 2.17 | $0.70 \times 10^{-3}$ |
| 90 | 5.40 | 3.33 | 4.93 | 7.2 | 2.4 | $0.80 \times 10^{-3}$ |
| 120 | 5.50 | 4.17 | 4.46 | 7.2 | 2.33 | $0.77 \times 10^{-3}$ |
| (No $Na^+$ added at 30 min) | | | | | | |
| 60 | 5.40 | 1.9 | 2.32 | 7.2 | 2.17 | $0.7 \times 10^{-3}$ |
| 90 | 5.40 | 2.27 | 2.9 | 7.2 | nd | nd |
| 120 | 5.50 | 2.5 | 2.09 | 7.2 | nd | nd |

Values for matrix pH in the presence of MgATP were taken from Fig. 5.6. Total matrix $Ca^{2+}$(mM) was calculated from the value of the $Ca^{2+}$ content (nmol.$mg^{-1}$) at each time point (Fig. 5.5) and assuming an internal volume of 3μl.$mg^{-1}$ protein. Free $Ca^{2+}$ (μM) was calculated from matrix pH, total matrix $Ca^{2+}$ and total matrix EDTA (10 mM), using the method of Storer and Cornish-Bowden (1976). n.d., not determined.

### *5.3 Discussion*

I have used $Na^+$-loaded resealed membrane "ghosts" to assay direct $Ca^{2+}/Na^+$ exchange activity across the chromaffin granule membrane: $^{45}Ca^{2+}$ accumulation was monitored in response to an outwardly-directed $Na^+$ gradient generated by dilution of $Na^+$-containing "ghosts" into a $Na^+$-free medium. Free $Ca^{2+}$ concentrations in the micromolar range were provided by $Ca^{2+}$/EGTA and $Ca^{2+}$/HEDTA buffers and were measured in uptake media at pH 7.0 using a sensitive $Ca^{2+}$-selective minielectrode. Following dilution, uptake was terminated after a few seconds by rapid addition of ice-cold quench medium containing EGTA, and $Ca^{2+}$ accumulated within the "ghost" matrix was quantitated using filtration and scintillation counting. By measuring initial rates of uptake, the apparent $K_m$ for $Ca^{2+}$ during $Ca^{2+}/Na^+$ exchange is between 1 and 3μM , with $V_{max}$ values of 3.5-8 nmoles.$mg^{-1}$.4 $sec^{-1}$ (equivalent to 50-120 nmol.$mg^{-1}$.$min^{-1}$being found, depending on the chelating agent present in the uptake media. Krieger-Brauer & Gratzl (1983) and Saemark *et al.,* (1983a) obtained values for the $K_m$ for $Ca^{2+}$ during $Ca^{2+}/Na^+$ exchange in chromaffin and neurohypophyseal membrane vesicles of 0.3 and 0.7μM, respectively. These data were obtained using $Ca^{2+}$/EGTA buffers and are similar to the value of 1.0μM determined using $Ca^{2+}$/EGTA solutions in this study. However, their values for $V_{max}$ were much lower: 14.5 and 1.5nmol.$mg^{-1}$.$min^{-1}$ for chromaffin and neurohypophyseal vesicles, presumably because initial rates of $Ca^{2+}$ uptake could not be measure accurately. For comparison, the $V_{max}$ of the $Na^+/H^+$ antiporter, determined from $^{22}Na^+$ transport rates in pH jump experiments in the absence of ATP (see Chapter 4 ) has been estimated to be of the order of 150-200 nmols.$mg^{-1}$ $min^{-1}$ , with initial rates sustained for only a few seconds presumably due to the rapid dissipation of the transmembrane pH gradient. The $V_{max}$ of the $H^+$-translocating ATPase of the granule membrane is about 100-200 nmols.$mg^{-1}$ .$min^{-1}$ (Johnson *et al.,* 1982a; Percy *et al.,* 1985) and that of the catecholamine transporter is about 15nmol.$mg^{-1}$ .$min^{-1}$ (Carty *et al.,* 1985).

Investigations of $Ca^{2+}/Na^{+}$ exchange have been greatly aided in recent years by the use of isolated resealed membrane vesicles (mainly derived from plasma membranes). The best-characterised system is that from heart sarcolemma, in which initial rates for $Na^{+}_{i}$-dependent $Ca^{2+}$ uptake of up to 20-30 $nmol.mg^{-1}sec^{-1}$ have been measured (Philipson *et al.,* 1982; Caroni & Carafoli, 1983). The apparent $K_m$ ($Ca^{2+}$) for the initial rate of $Na^{+}_{i}$-dependent $Ca^{2+}$ uptake in cardiac sarcolemmal vesicles is generally in the range 15-40μM (Bersohn *et al.,* 1982; Reeves & Sutko, 1983; Slaughter *et al.,* 1988) although Caroni & Carafoli (1983) obtained a more physiologically acceptable value of 2μM.

However, the determination of $K_m$ and $V_{max}$ values in these sorts of preparations are complicated by a number of factors. EGTA and several other well known chelating agents are often used to control the free $Ca^{2+}$ concentrations within the physiological range; experimental findings are generally interpreted on the basis that EGTA, or indeed its liganded form, $Ca^{2+}$-EGTA, is biologically inert. In several well-characterised $Ca^{2+}$ transport systems, EGTA or one of its liganded species exerts an activating effect. For example, Al-Jobore & Roufogalis (1981) have observed stimulation by EGTA of the ($Ca^{2+}$,$Mg^{2+}$)-ATPase of human erythrocyte membranes. Birch-Machin & Dawson (1986), in a detailed study on the $Ca^{2+}$-ATPase of rat liver plasma membranes, found that EGTA or one of its liganded species activated the enzyme; activation was dependent on the free $Ca^{2+}$ concentration and was not an artefact of the isolation procedure. Moreover, the stimulatory effect of EGTA was not apparent at free $Mg^{2+}$ concentrations of 10μM or more. Other widely used chelating agents such as EDTA and HEDTA did not exert this activating effect on the enzyme.

In studies of $Ca^{2+}/Na^{+}$ exchange by isolated cardiac sarcolemmal vesicles, the affinity of the carrier for $Ca^{2+}$ can be markedly increased using EGTA or EDTA. Apparently, as little as 3μM was effective, and stimulation of $Na^{+}_{i}$-dependent $Ca^{2+}$ uptake by an order of magnitude or more occurred as the chelator concentration was increased $\geq$ 20μM (Trosper & Philipson, 1984).

In the $Na^+$-loaded "ghost" preparation used here both the measured $K_m$ and $V_{max}$ values are different depending on the chelating agent used: a higher affinity (lower $K_m$) and higher $V_{max}$ are obtained using EGTA compared to HEDTA. However, one cannot conclude from these data whether or not EGTA is having an "activating" effect on the system, as opposed to an "inhibitory" effect of HEDTA.

At the present time there is insufficient evidence to support a particular hypothesis of how EGTA and other $Ca^{2+}$ chelators increase the activity of a number of $Ca^{2+}$ transport processes. The chelated complexes may interact directly with particular ATPases or exchangers to alter their $Ca^{2+}$ binding or transport properties, and may mimic the effect of naturally-occurring cytosolic $Ca^{2+}$ ligands. It is well-known that the ubiquitous $Ca^{2+}$ binding protein calmodulin directly stimulates the $Ca^{2+}$-ATPase of several plasma membrane types, increasing the $Ca^{2+}$ affinity of the enzyme (from > 10μM to < 1μM) and its rate of transport of $Ca^{2+}$ (Jarrett & Penniston, 1977; Niggli *et al.,* 1981). A large number of cytosolic $Ca^{2+}$-binding proteins, in addition to calmodulin are present in several secretory cells (including chromaffin cells, where they are known as "chromobindins"; see Table1.1, p. 16) and may play a significant role in $Ca^{2+}$-dependent exocytosis (Creutz *et al.,* 1987; 1988). However, the binding of "chromobindins" to resealed chromaffin granule "ghosts" in the presence of extravesicular $Ca^{2+}$ concentrations in the range 5-40μM appears to have no effect on granule membrane calcium transport (J.H. Phillips, unpublished work).

Most $Ca^{2+}/Na^+$ exchange experiments of the type described here are designed to maximise transport activity and to afford a quantitative definition of the kinetic parameters, uncomplicated by other events involving the two cations. For convenience, $Na^+$ and $Ca^{2+}$ are initially present on opposite sides of the membrane. However, *in vivo*, $Na^+$ and $Ca^{2+}$ are present in both chromaffin vesicle matrix and cytosol and the relatively high $V_{max}$ values measured *in vitro* may not apply. Extravesicular $Na^+$ non-competitively inhibits $Ca^{2+}$ uptake in "ghosts" (Phillips, 1981) and intact granules (see Chapter 3). In addition, Ca2+/Na+ exchange may

be pH-sensitive, as it is in cardiac sarcolemmal vesicles (Philipson *et al.,* 1982; Slaughter *et al.,* 1983). Furthermore, $Ca^{2+}/Na^{+}$ exchange is complicated because the "ghost" preparation used here (Apps *et al.,* 1980) contains a small proportion of inside-out (i.e. matrix face cytoplasmically orientated) vesicles mixed with the correctly sealed vesicles, as well as a proportion of broken membranes (Hunter *et al.,* 1982). Resealed vesicles (of either orientation) will participate in $Ca^{2+}/Na^{+}$ exchange reactions but interpretation of the data is not easy because the cation binding sites on either face may have differing affinities.

Nevertheless, the apparent $K_m$ is sufficiently low to envisage the exchanger being of physiological importance, responsive to the low free $Ca^{2+}$ concentrations (0.1 - 0.2μM) that exist in the cytosol. That the uptake of $Ca^{2+}$ into the matrix is dependent on the activity of the $H^{+}$-translocating ATPase and the $Na^{+}/H^{+}$ antiporter is demonstrated by the results shown in Fig. 5.5. This result, notwithstanding some uncertainties, is an important one because it lends support to the hypothesis that $Ca^{2+}$ regulation in the adrenal medulla may involve active accumulation of $Ca^{2+}$ into the granule matrix, followed by eventual loss from the cell during exocytosis; accumulation can be achieved not by direct coupling of $Ca^{2+}$ influx to ATP hydrolysis (involving a $Ca^{2+}$-translocating ATPase) but by coupling to a transmembrane $Na^{+}$ gradient, which ***is*** dependent on ATP hydrolysis and inwardly directed electrogenic proton translocation. Under conditions where an intragranular pH of about 5.5 is being sustained, and in the presence of externally buffered $Ca^{2+}$, very little $Na^{+}$ appears to be accumulated within the matrix. However, under almost identical conditions in the absence of external $Ca^{2+}$, an 8-fold $Na^{+}$ concentration gradient (inside minus outside) could be established (Chapter 4, Fig. 4.2).

Because the internal $Na^{+}$ concentration in these experiments with "ghosts" remains very low, measured rates of $Ca^{2+}$ accumulation are also very low under conditions that are designed to mimic the environment of a granule in the cytosol of a chromaffin cell. Indeed, the level of free $Ca^{2+}$ attained (4.7μM, Table 5.2) is very

similar to the measured free $Ca^{2+}$ concentration in the matrix of isolated intact chromaffin granules (described in the next chapter).

Therefore, there may be an additional route of $Na^{+}$ efflux from the granules, possibly involving a cation-conducting channel in the granule membrane, that has yet to be characterised. Under such circumstances granules seem unlikely to play a major role in calcium homeostasis.

# Chapter Six

# Free concentrations of $Na^+$, $K^+$, and $Ca^{2+}$ in Chromaffin Granules

## *6.1 Introduction*

In addition to storing high concentrations of hormones and neurotransmitters, many exocrine and endocrine secretory granules contain high concentrations of divalent cations (see Table 1.3, page 37) the function of which is still rather speculative. In some granules, such as the chromaffin granules of the adrenal medulla and neurohypophyseal vesicles of the pituitary, membrane transport mechanisms have been identified which may prove to be important in cation homeostasis in the secretory cell. The large concentration of $Ca^{2+}$ in some granules may represent a potentially mobilisable pool *in vivo*. Alternatively, divalent cations may have a role in the early stages of granule biogenesis, promoting aggregation of soluble constituents such as proteins and nucleotides (Winkler, 1977).

The total concentration of divalent cations in difference secretory granules is rather variable. In the rat, parotid granules contain 8mM $Ca^{2+}$ and 5mM $Mg^{2+}$ (Castle *et al.*, 1987), whereas insulinoma granules contain exceedingly high concentrations: 120mM and 72mM, respectively (Hutton *et al.*, 1983). In the chromaffin granule $Ca^{2+}$ and $Mg^{2+}$ have been measured by atomic absorption spectrophotometry, giving nominal total concentrations of 20mM and 5mM, respectively (Borowitz, 1965; Phillips *et al.*, 1977). Recently, electron probe microanalysis of granules in cultured chromaffin cells (Ornberg *et al.*, 1988) yielded values of 8.8mM $Ca^{2+}$ and 5mM $Mg^{2+}$. However, as most secretory granules contain high concentrations of adenine nucleotides (such as ATP) and negatively charged proteins, divalent cations are expected to be largely bound, leaving only a small fraction free within the matrix.

Free concentrations of $Ca^{2+}$ and $Mg^{2+}$ have only been measured in a few instances; in the $\alpha$-granules of platelets values of 12 and 326$\mu$M, respectively, were found. These values are markedly lower than the calculated total concentrations of 32mM for $Ca^{2+}$ and 172mM for $Mg^{2+}$ (Grinstein, *et al.*, 1983). In chromaffin granules, Bulenda & Gratzl (1985) estimated the free matrix $Ca^{2+}$ concentration to be

between 4μM and 24μM using $Ca^{2+}$-specific electrode to detect $Ca^{2+}$ fluxes in the presence of the ionophore A23187. While these values seem reasonable, the measured matrix pH, at 6.2, was rather high (most workers agree on values between 5.5 and 5.7); in addition, the use of the ionophore may have affected the ion specificity of the electrode. Bulenda & Gratzl (1985) were unable to measure the free concentration of matrix $Mg^{2+}$.

To date, much less attention has been paid to the common monovalent cations, such as $Na^+$ and $K^+$. As for divalent cations, results seem rather variable: rat parotid granules contain 4mM $Na^+$ and 2mM $K^+$ (Castle *et al.*, 1987) whilst rat insulinoma granules appear to contain much higher levels (29mM $Na^+$ and 16mM $K^+$; Hutton *et al.*, 1983). In isolated chromaffin granules, Salama *et al.*, (1980), using the fluorescent pH indicator 9-aminoacridine and the $K^+/H^+$ exchange ionophore nigericin concluded that intragranular free $K^+$ was about 2mM. Krieger-Brauer & Gratzl (1982), using flame photometry, estimated intragranular $Na^+$ to be about 47mM. Analysis of chromaffin granules from a human adrenal medulla tumour (pheochromocytoma) gave values of about 22mM each for $Na^+$ and $K^+$ (Johnson *et al.*, 1982b). Using the electron probe microanalysis method, Ornberg *et al.*, (1988) found values of OmM $Na^+$ and 83mM $K^+$ for granules in cultured chromaffin cells. Interestingly, these authors showed that the monovalent and divalent cation content of granules isolated from intact glands was found to vary according to the ionic composition of the buffer used for isolation. In MES/NaOH buffer the values were (in mM) $Ca^{2+}$, 7.0; $Mg^{2+}$, 0; $Na^+$, 29 and $K^+$, 19. In MES/KOH buffer they were 12, 0, 8 and 40mM, respectively.

The total concentrations of cations have been estimated from measurements of cation content and the intragranular water space. However, in most cases, with the exception of $H^+$, the free concentrations of cations have not been determined although this is clearly important for an understanding of bioenergetic processes involving these ions. Recently, null point titration techniques using cation exchanging ionophores combined with measurements of pH fluxes have been used successfully to estimate the free concentrations of $Ca^{2+}$, $Mg^{2+}$, $Na^+$ and $K^+$ in a number of cells.

In this chapter, I present a method for estimating the free concentrations of $Ca^{2+}$, $Na^{+}$ and $K^{+}$ in isolated chromaffin granules; a fluorimetric method using the weak base 9-amino-6-chloro-2-methoxyacridine (ACMA) to monitor intragranular pH changes in the presence of ionophores has been employed. For comparison, the total concentrations of $Na^{+}$ and $K^{+}$ were also determined using flame photometry. These results show that $Ca^{2+}$ is largely bound within the granule matrix, whereas most of the matrix $Na^{+}$ and $K^{+}$ remains free. The results also show that monovalent ion redistribution occurs during granule isolation and therefore, the concentrations of $Na^{+}$, $K^{+}$ and $Ca^{2+}$ measured *in vitro* cannot be extrapolated to the intact cell *in situ.*

## *6.2 Results*

In the experiments described here ACMA was used with cation exchanging ionophores to estimate the free concentrations of $Na^+$, $K^+$ and $Ca^{2+}$ in the granule matrix. An account of the theory of ACMA quenching and its use with chromaffin granules is described in Chapter 2.

### *6.2.1 Granule Purity*

Chromaffin granules may be purified by differential centrifugation. However, this makes them fragile, so for the purpose of these measurements pellets of granules were simply washed vigorously with buffered sucrose to remove overlying mitochondria, which were discarded. Mitochondrial contamination of the granule preparations was assessed using cytochrome c. oxidase; it was found to be equivalent to 4.5 + 0.8% (S.E.M. for five preparations of granules) of the protein content.

### *6.2.2 Internal pH of Chromaffin Granules*

[$^{14}$C]methylamine was used to determine the internal pH of freshly isolated chromaffin granules at a variety of external pH values. On 4 different granule preparations the internal pH ($pH_i$) was 5.53 $\pm$ 0.07 (mean $\pm$ S.E.M.), results identical with those of Johnson and Scarpa (1976b).

I then measured the quenching of ACMA fluorescence by granules in solutions at a variety of pH values ranging from pH 5.85 to pH 7.72 and the results were plotted as follows:

$$\log [Q/(100-Q)] = \Delta pH - \log (v_o/v_i) \qquad (1)$$

as described in Chapter 2. Fig.6.1 shows that a good straight line is obtained, with a

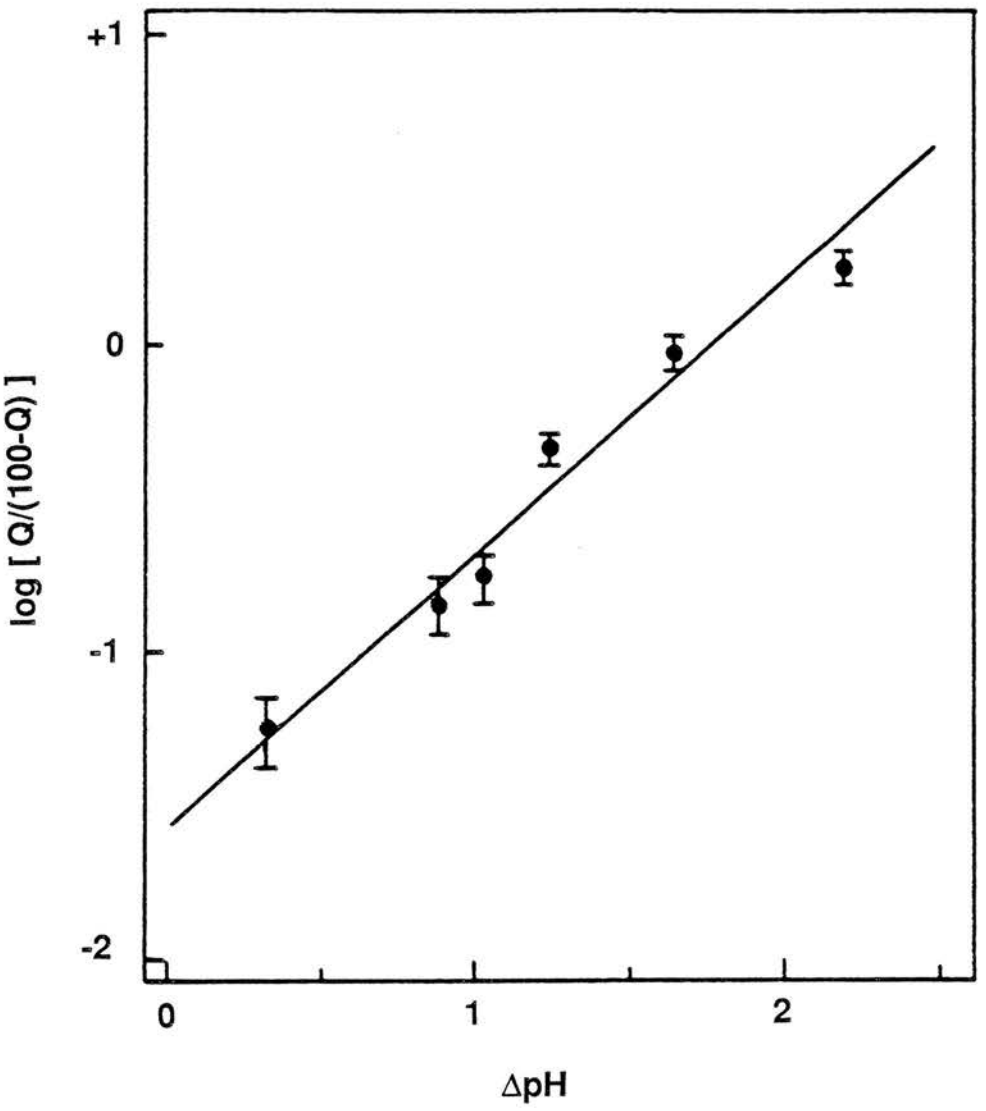



***Fig. 6.1 ACMA fluorescence quenching by chromaffin granules***

Chromaffin granules (0.10 $mg.ml^{-1}$) were added to media buffered at various pH values, and ACMA fluorescence quenching (Q) was measured. Values are means ± S.E.M. for triplicate determinations.

slope of 0.85 and a y-intercept (log [Q/100-Q] axis) of -1.53. At the y-intercept $\Delta pH = 0$; therefore this value is equal to -log ($v_o/v_i$), giving a notional value for $v_o/v_i$ of 33. Using a value for the internal volume for chromaffin granules suspended in buffered 0.3M sucrose of 4.3$\mu$l.mg$^{-1}$ protein (Pollard *et al.*, 1976; Phillips *et al.*, 1977), the actual value of the ratio $v_o/v_i$ is approximately 2300. As observed previously from fluorescence experiments with resealed "ghosts" (Chapter 4), the deviation of both slope and intercept from ideal values is probably due to some binding of ACMA to the granule membranes (Warnock *et al.*, 1982).

In all subsequent experiments with ACMA, media were buffered at pH 6.5 and $\Delta pH$ was assumed to be 1.00. This gives around 20% quenching of ACMA fluorescence at the protein concentrations used.

### *6.2.3 Ion Content of Granules*

The results of Ornberg *et al* ., (1988) suggested that the monovalent ion content of granules is highly dependent on the ionic composition of the isolation buffer, and that monovalent ions may be expected to exchange with ions in the isolation medium. Indeed, Ornberg *et al* ., (1988) found that granules isolated from cultured cells were devoid of $Na^+$, but had a high content of $K^+$.

In this work, trimmed adrenal glands have been placed on ice within one hour of slaughter; it is clearly possible that such ion re-equilibration may have taken place. Granules were then prepared in ice-cold sucrose media buffered with Hepes neutralised with NaOH, KOH or tetramethyl ammonium (TMA) hydroxide. After washing the granules with sucrose buffered with Hepes-TMA, concentrated suspensions were lysed and their $Na^+$ and $K^+$ contents determined by flame photometry (Table 6.1). It can be seen that the $K^+$ content is identical in granules isolated in Hepes/NaOH and Hepes/TMA, although it is higher when Hepes/KOH was used. The $Na^+$ content is slightly lower in granules isolated in Hepes/KOH compared with Hepes/TMA; however, the $Na^+$ content of granules prepared in Hepes/NaOH is more than double that

***Table 6.1. $Na^+$ and $K^+$ contents of chromaffin granules***

Chromaffin granules were prepared in the isolation buffer shown and $Na^+$ and $K^+$ contents were measured by flame photometry. Internal concentrations were calculated using an internal volume of 4.3µl.mg$^{-1}$ (Phillips *et al.*, 1977). Values given are means ± S.E.M. for triplicate determinations on the number of granule preparations shown (n).

| **Isolation buffer** | **HEPES/NaOH** | | **HEPES/KOH** | | **HEPES/TMA** | |
|---|---|---|---|---|---|---|
| | **$Na^+$** | **$K^+$** | **$Na^+$** | **$K^+$** | **$Na^+$** | **$K^+$** |
| Ion content (nmol.mg$^{-1}$) | 156 | 127 | 75 | 179 | 99 | 128 |
| | ±11.5 | ±7.8 | ±10.5 | ±12.3 | ±10.6 | ±6.9 |
| n | 6 | 6 | 5 | 6 | 5 | 5 |
| Internal concentration (mM) | 36.2 | 29.5 | 17.4 | 41.6 | 23.0 | 29.7 |
| Sum of $[Na^+]_i$ + $[K^+]_i$ (mM) | 65.7 | | 59.0 | | 52.7 | |

present in Hepes/KOH granules. The combined total concentration of these monovalent ions was 50-60mM and, in agreement with the results of Ornberg *et al.*, (1988), appeared to reflect the ionic composition of the medium used for granule isolation.

### *6.2.4 Free Concentrations of Ions in the Granule Matrix*

For the determination of free ion concentrations only granules prepared in media buffered with Hepes/TMA were used. The approach is based on ionophore mediated electroneutral fluxes of $H^+$ and the particular cation ($M^{x+}$) under consideration, as described in Chapter 2.

In these experiments I have used the ionophore nigericin for measuring $K^+$ fluxes, as well as monensin for $Na^+$ and A23187 for $Ca^{2+}$. I will describe an experiment using nigericin to determine the null point and so calculate $[K^+]_i$. In Fig. 6.2 a suspension of granules is added to a solution containing ACMA, 2mM $Na^+$ and buffered at pH 6.5. There is an immediate quenching (Q) of ACMA fluorescence, the extent of which depends on the size of the $\Delta$pH, as given by eqn. (1), described previously.

On addition of nigericin to the medium, there is a movement of $K^+$ ions and a compensatory flux of protons as equilibrium is established. This is reflected by an increase or decrease of ACMA quenching: in Fig. 6.2 there is a further quenching in the absence of external $K^+$, and a decreased quenching in the presence of 8mM $K^+$. At equilibrium, the new percentage of quenching is known as $Q^*$ At this point:

$$[K^+]_i / [K^+]_o = [H^+]_i / [H^+]_o = [ACMA]_i / [ACMA]_o \qquad (2)$$

and, by analogy with Eqn (1),

$$\log [Q^*/(100-Q^*)] = -\log [K^+]_o + \log [K^+]_i - \log (v_o/v_i) \qquad (3)$$

The volume ratio is constant. For small variations in $[K^+]_o$ there will be a small

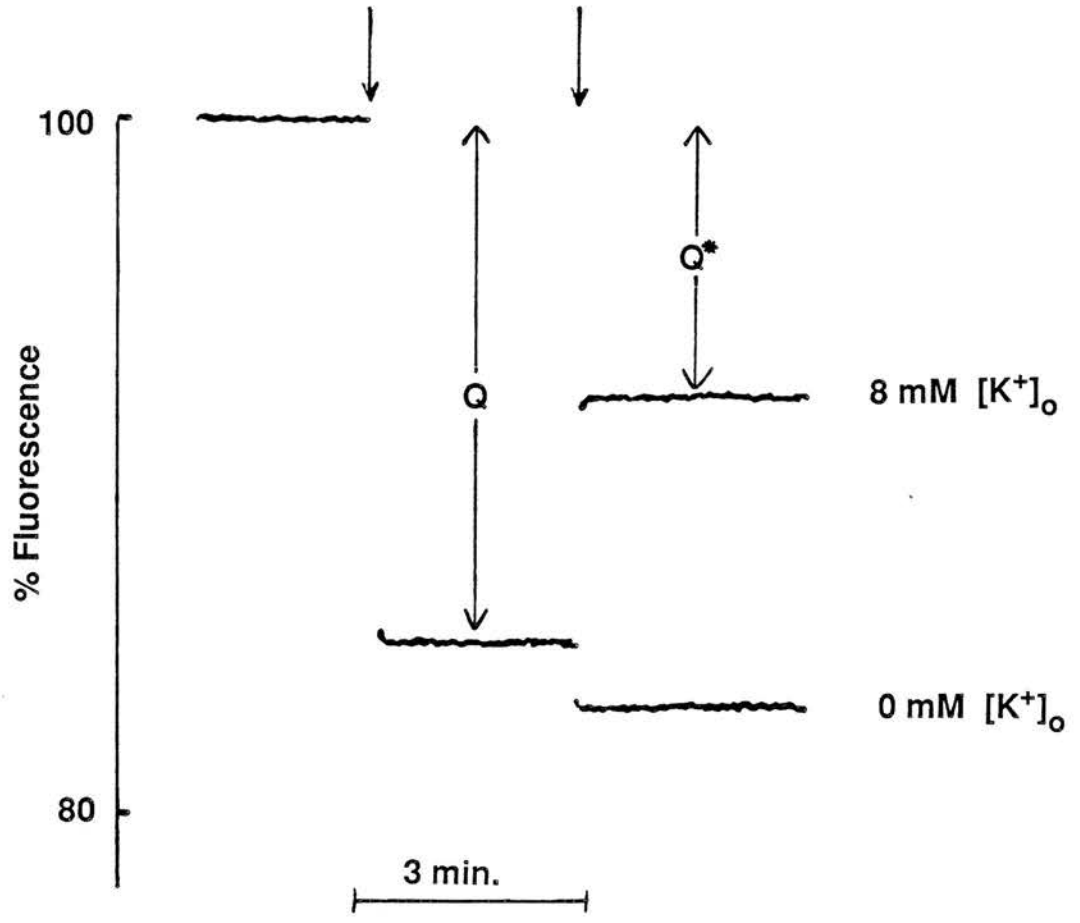



***Fig. 6.2 ACMA fluorescence with chromaffin granules***

Fluorescence (arbitrary units) was recorded continuously. At the first arrow a suspension of granules was added to give a protein concentration of 0.09 $mg.ml^{-1}$. The percentage quenching that results is referred to as Q in the text. At the second arrow, nigericin is added. Examples are shown for a medium that is free of $K^+$, or one that contains 8mM $K^+$. The new value of percentage quenching is referred to as $Q^*$ in the text.

variation in $[K^+]_i$, but this will be approximately proportional to $[K^+]_o$, so that a plot of log $[Q^*/(100-Q^*)]$ against log $[K^+]_o$ should be a straight line. The null point may be described as that value of $[K^+]_o$ that produces no change in ACMA quenching on addition of ionophore. Here $Q = Q^*$ and $pH_i$ is unchanged. Knowing $pH_i$, $pH_o$ and $[K^+]_o$, $[K^+]_i$ can be calculated from eqn. (2) above.

The null points for $Na^+$ using monensin and $Ca^{2+}$ using A23187 were determined in the same way. For a particular batch of granules, I determined the null point for $K^+$ using nigericin first, and then added this concentration of $K^+$ to media used in experiments with monensin in order to minimise $K^+$ fluxes during study of $Na^+$. $Mg^{2+}$ fluxes did not interfere with studies on $Ca^{2+}$ when A23187 was used (see below).

A typical experiment for the determination of the null point for $K^+$ is shown in Fig. 6.3a, with the results plotted according to eqn. (3). A good straight line is obtained over the range of $[K^+]_o$ used (1.5 to 6.0mM), with a slope of 0.91 (theoretical value 1.0), suggesting rather little change in $[K^+]_i$ over this range. Quenching after ionophore addition in this experiment gave values for $Q^*$ of 7.4 to 22.5%; the null point is then found by averaging values for Q and then determining the value of $[K^+]_o$, as shown in the figure (2.45mM in this case). Steady fluorescence traces were obtained within about 30 sec of adding the ionophore. All solutions contained 2mM $Na^+$, the approximate value of the null point for $Na^+$. Under these conditions the $Na^+$ and $H^+$ gradients are in equilibrium across the membrane, as follows:

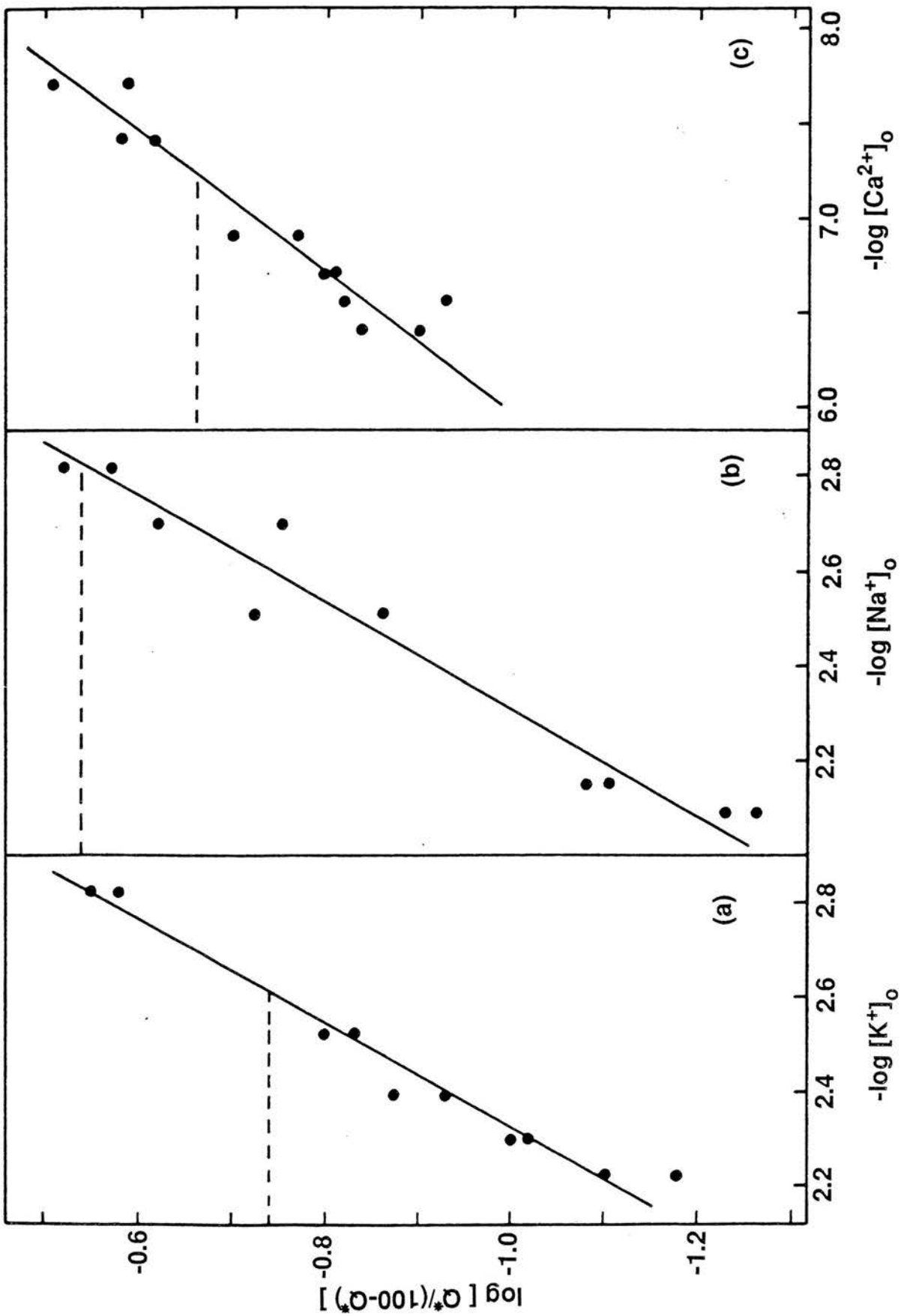
$$[Na^+]_i / [Na^+]_o = [H^+]_i / [H^+]_o \qquad (4)$$

Therefore, the activity of the $Na^+/H^+$ antiporter in the granule membrane described in Chapter 4 does not interfere with $K^+$ determinations using nigericin.

Similar experiments were then performed on the same batch of granules in order to determine the null point for $Na^+$ using monensin (Fig. 6.3b). These media contained the null point concentration of $K^+$ just measured, in order to minimise a

***Fig. 6.3 Null-point determination for $K^+$, $Na^+$ and $Ca^{2+}$***

**(a)** Quenching of ACMA fluorescence ($Q^*$) by granules (0.09 $mg.ml^{-1}$) was measured in the presence of various concentrations of $K^+$, with all readings made in duplicate. The mean value of quenching before ionophore addition (Q) was 15.4 ± 0.6% (± S.E.M., n = 10). This is indicated by the interrupted line on the Figure. **(b)** As **(a)**, except that $[Na^+]_o$ was varied. Granule concentration was 0.07 $mg.ml^{-1}$ and the mean value of Q was 22.5 ± 1.0% (n = 10). **(c)** As **(a)**, except that $[Ca^{2+}]_o$ was varied. Granule concentration was 0.07 $mg.ml^{-1}$ and the mean value of Q was 18.2 ± 1.1% (n = 14). All concentrations are molar.


log [ Q*/(100-Q*) ]
-0.6
-0.8
-1.0
-1.2
(a)
(b)
(c)
2.2
2.4
2.6
2.8
-log [K+]o
-log [Na+]o
6.0
7.0
8.0
-log [Ca2+]o

contribution from $K^+$ fluxes. Again, a good straight line was obtained with a slope of 0.88 over the range of $[Na^+]_o$ used (1.5 to 6.0mM). In Fig. 6.3b the null point is found at 1.47mM $Na^+$.

Finally, $Ca^{2+}$ was investigated using A23187. External $Ca^{2+}$ concentrations between 20nM and 400 nM were maintained using a $Ca^{2+}$-EGTA buffer at pH 6.5 and the line obtained (Fig. 6.3c) had a slope of 0.27. The deviation from the predicted value (0.5 for a divalent cation) is more than that observed with $K^+$ and $Na^+$, in spite of $Ca^{2+}$ buffering inside the granule which is expected to reduce variation in $[Ca^{2+}]_i$. Nevertheless, a null point value for $[Ca^{2+}]_o$ of 56nM was obtained (Fig. 6.3c ).

An attempt was made to measure free $Mg^{2+}$ concentrations within the matrix using A23187. A series of $Mg^{2+}$-EDTA buffers were used, giving free external $Mg^{2+}$ concentrations within the range 20-120nM; in each solution free $Ca^{2+}$ was held constant at 63nM (a value similar to the null point $Ca^{2+}$ concentration). However, no fluorescence changes were observed on addition of the ionophore, and I conclude (as did Bulenda and Gratzl,1985) that $Mg^{2+}$ movements are negligible under these conditions.

Table 6.2 shows the results of these free ion concentration measurements. The null points and the range of slopes found in experiments with different batches of granules are given. The free concentrations of these ions calculated from these data are also presented. Comparison of the total internal $Na^+$ and $K^+$ concentrations for Hepes/TMA granules given in Table 6.1, and the calculated free $Na^+$ and $K^+$ concentration for these granules in Table 6.2, allows one to estimate the matrix activity coefficients for these ions. The values are 0.81 for $Na^+$ and 0.77 for $K^+$, indicating that these ions are largely free within the matrix. In contrast, the free concentration of $Ca^{2+}$ in the matrix (5.6μM, Table 6.2) is markedly lower than the previously measured total concentration of 20mM (Phillips *et al.*, 1977). In other words, its free concentration is only 0.03% of the total. Because of the errors in the determination of ΔpH mentioned in Chapter 2 these activity coefficients are only approximate.

***Table 6.2. Free ion concentrations of the chromaffin granule matrix***

Chromaffin granules were prepared in HEPES/TMA buffered sucrose. Null points were determined as described in Fig. 6.3. Results are means ± S.E.M. for the number of granule preparations shown (n). Slopes are given for plots as in Fig. 6.3. The free ion concentration is calculated using ΔpH = 1.0.

| | $Na^+$ | $K^+$ | $Ca^{2+}$ |
|---|---|---|---|
| Null point concentration | 1.88 mM (± 0.17) | 2.28mM (± 0.13) | 0.056 μM (±0.0064) |
| n | 4 | 5 | 4 |
| Range of slopes | 0.82-0.90 | 0.72-0.91 | 0.24-0.35 |
| Free internal concentration | 18.8mM | 22.8mM | 5.6μM |

### *6.3 Discussion*

I have described a simple method for determining the free ion concentrations inside acidic organelles. A rather similar method has been described by Grinstein *et al.*, (1983) for platelet α-granules using 9-aminoacridine (9-AA) as the fluorescent dye. However, ACMA (used in this study) is more suitable because of its very rapid response times: fluorescence quenching is immediate and steady traces are obtained within 30 seconds. In their experiments, Grinstein *et al.*, (1983) estimated free $Ca^{2+}$ and $Mg^{2+}$ concentrations by employing a double null-point titration using A23187 and the halogenated analogue 4-bromo-A23187 (Br-A23187). These ionophores have different $Ca^{2+}/Mg^{2+}$ selectivity ratios and free concentrations of 12μM $Ca^{2+}$ and 326μM $Mg^{2+}$ were found.

A preparation of organelles made by subcellular fractionation is never pure; I purified granules as completely as was possible while providing intact granules rapidly. Mitochondrial contamination can be assessed accurately, using purified mitochondria as a standard. This was found to be relatively low and I have calculated that mitochondrial $K^+$ (measured by Ornberg *et al.*, 1988) contributed only 2 or 3% to the measured content of the granule preparation (Table 6.1). I therefore ignored ACMA movements induced by the addition of nigericin to, and resultant acidification of, these organelles.

The granules may contain a relatively high proportion of adrenal lysosomes (perhaps 25%; see Phillips, 1973) but the extent of contamination of the granules in terms of percentage of their protein cannot be assessed accurately in the absence of "pure" adrenal lysosomal material. The adrenal content of lysosomal protein is low compared with its massive content of granule protein (Smith & Winkler, 1966), and I have, again, ignored this.

ACMA quenching by lysosomes in the suspension will be little affected by ionophore addition, thus appearing to reduce the change in quench that is observed. Such errors contribute to the non-ideality of the responses that is seen in Fig. 6.3 and recorded in Table 6.2. These errors are not easily quantitated, but they are likely to be small.

My value for the free $Ca^{2+}$ concentration (5.6μM) agrees closely with one of the values given by Bulenda & Gratzl (1985), a value they obtained when the pH gradient across the granule membrane was collapsed using $NH_4Cl$. Atomic absorption analyses of granules gives values for the $Ca^{2+}$ content of 77-100 nmoles.$mg^{-1}$ (Borowitz 1967; Phillips *et al.*,1977), or a nominal concentration of around 20mM. The concentration of free $Ca^{2+}$ in the matrix is therefore about 0.03% of the total content, a value almost identical to that found in platelet α-granules (Grinstein *et al.*, 1983). I was unable to detect $Mg^{2+}$ fluxes in granules using A23817. I had expected to find a lower free $[Mg^{2+}]_i$ than $[Ca^{2+}]_i$, but anticipated being able to follow $Mg^{2+}$ fluxes induced by A23187 if $[Ca^{2+}]_o$ was set to its null point value.

However, the value of Ornberg *et al.*, (1988) for total $Mg^{2+}$ (5mM) is identical to that measured by Phillips *et al.*, (1977) using atomic absorption spectrophotometry, although $Mg^{2+}$ appeared to be totally excluded from granules isolated in MES/NaOH and MES/KOH buffered solutions. At present therefore, some query must remain over the $Mg^{2+}$ status of chromaffin granules. Moreover, the value of 8.8mM total $Ca^{2+}$ measured in granules in cultured adrenal cells using electron probe microanalysis (Ornberg *et al.*, 1988) is rather low. This was attributed to a difference in the water content used to calculate wet concentrations.

Total contents of $Na^+$ and $K^+$ varied between 227 and 283 nmol.$mg^{-1}$ of protein, equivalent to matrix concentrations of 53 to 66mM (Table 6.1). The value found depended on the isolation buffer used, and the ratio of the two ions reflected the ionic composition of the buffer.

The values presented here are similar to those of Ornberg *et al.,* (1988), but different from previous values for total intragranular $Na^+$ of 30-50m M

(Krieger-Brauer & Gratzl, 1982) and $K^+$ of 2mM (Salama *et al.*, 1980). In neurosecretory vesicles isolated from the posterior pituitary (Russell, 1984) a value for intragranular $K^+$ of 0.75mM was determined.

The activity coefficients for $Na^+$ and $K^+$ of about 0.8 determined from the free concentrations (Table 6.2), indicate that most of these ions are free within the matrix; thus, these ions are presumably able to exchange fairly freely with ions in the external medium. Therefore, such ion redistribution as occurs when granules are isolated in different buffers, is not unexpected. Comparison of the different panels in Fig. 6.3 shows that the magnitude of percentage quenched after ionophore addition is about the same for the three ions. Therefore, it seems likely that the presence of a large reservoir of exchangeable $Ca^{2+}$ is required for an accurate null point determination, given that almost all of the $Ca^{2+}$ is bound whereas most of the $Na^+$ and $K^+$ remains free. Given the large internal pH buffering capacity of the granules (300μmol $H^+$ per pH unit per gram dry weight), then if only the free $Ca^{2+}$ were exchangeable, the ionophore induced ΔpH would be small and undetectable.

The precise nature of intragranular binding sites for divalent cations is unknown, although it is clear that within the matrix the behaviour of catecholamine and nucleotide molecules is far from ideal. At 37°C solutions containing 0.6M adrenaline and 0.15M ATP show an effective osmotic pressure of only 0.25 osM, and this value varies rather little with changes in ATP concentration above this value (Koppell & Westhead, 1982). However, subsequent addition of inorganic ions $Na^+$, $K^+$, $Cl^-$ and $Ca^{2+}$ to catecholamine/ATP mixtures increases the osmotic pressure in an almost ideal manner. This is consistent with the relatively high activity coefficients for $Na^+$ and $K^+$ found in the present study.

The very low value for the free $Ca^{2+}$ concentration is indicative that most of the $Ca^{2+}$ is bound to acidic matrix chromogranins (Reiffen & Gratzl, 1986) and glycosaminoglycans, since the results of Koppell & Westhead (1982) suggest that little is bound to ATP. Nevertheless, it has been reported (Südhof, 1983) that in the absence of extragranular $Ca^{2+}$, A23187 causes depletion of matrix $Ca^{2+}$ in a

dose-dependent manner with concomitant destabilisation and osmotic lysis; the latter could be totally prevented if 10μM external $Ca^{2+}$ was present. There is little doubt that more detailed investigations are needed to determine whether or not divalent cations have an important role in the formation and maintainance of polymeric complexes of catecholamines and nucleotides in chromaffin granules.

The free $Ca^{2+}$ concentration reported here (5.6μM) is approximately 25-50 fold higher than that in the unstimulated chromaffin cell cytoplasm (0.1-0.2μM). However, the measured content of $Na^+$ and $K^+$ within the matrix of isolated granules gives very little information about the ion distribution *in vivo*, and thus how this $Ca^{2+}$ activity gradient is established. Intragranular $Na^+$ is certainly fairly low in granules that have not been exposed to external $Na^+$ or $K^+$ during the isolation procedure (see Table 6.1); indeed, granules may be devoid of matrix $Na^+$ *in situ* , as suggested by Ornberg *et al.,* (1988).

These authors argue that mature chromaffin granules are unlikely to sequester $Ca^{2+}$ during secretion, by a process of $Na^+/Ca^{2+}$ exchange; such an exchange mechanism may operate during granule recycling following exocytosis and serve to remove bulk extracellular $Na^+$. During subsequent granule refilling, ATP (an anion), is accumulated first and ion pairs may be formed with $Na^+$. Inorganic $Na^+$ could then be replaced by catecholamine provided the organelle remains osmotically stable.

Therefore it is important to discover how the content of these ions is regulated *in vivo* in order to determine their role in the bioenergetic processes of these secretory granules.

# *Chapter Seven*

# *Conclusions and Perspectives*

Chromaffin granules isolated from secretory cells of the mammalian adrenal medulla are the most extensively characterised neuroendocrine storage organelles; this is primarily because of the ease of preparation of highly pure material. Nevertheless, very similar properties with regard to hormone or neurotransmitter storage, membrane bioenergetics and secretory mechanisms, have recently been described in a number of other, generally less readily available storage granules (reviewed by Njus *et al.*, 1987a).

The chromaffin granule membrane is an active structure, exhibiting a variety of complex functions; as with most secretory vesicles, the energy for these activities is provided by a proton electrochemical gradient generated by a proton-translocating ATPase. This enzyme utilises cytosolic (extragranular) ATP and catalyses inwardly directed electrogenic proton translocation; in chromaffin granules the resulting proton and potential gradients are used as an energy source for catecholamine transport. Catecholamines are stored at high concentrations (0.55M) together with nucleotides (0.13M), a variety of acidic proteins and peptides, and inorganic ions such as $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$, and $K^{+}$. Although in some secretory granules divalent cations are present at very high concentrations, their role is still largely speculative. Transport processes for these cations have been described in several secretory vesicle membranes and, therefore, they may be involved in cation homeostasis in the secretory cell.

### *7.1 $Na^{+}$ Transport by Chromaffin Granule Membranes*

In this thesis, transport mechanisms for $Ca^{2+}$ and $Na^{+}$ have been described in intact granules and resealed "ghosts"; the most useful results have been obtained with "ghosts", due to their ability to accumulate large quantities of substrates, uncomplicated by the presence of endogenous matrix constituents which tend to leak

during incubations. An important goal at the beginning of this project was to elucidate the mechanism(s) of $Na^+$ transport in more detail, apart from $Ca^{2+}/Na^+$ exchange, which had been described previously (Phillips 1981; Krieger-Brauer & Gratzl, 1982, 1983).

Unlike mitochondria, which are endowed with independent uptake and efflux pathways for $Ca^{2+}$ (electrogenic uniport and electroneutral $Ca^{2+}/2Na^+$ or $Ca^{2+}/2H^+$ exchange), present evidence favours a single $Ca^{2+}$ transport system in chromaffin granules which would therefore be dependent on intragranular $Na^+$; to sustain $Ca^{2+}$ uptake, an additional $Na^+$ entry mechanism (independent of $Ca^{2+}$) is required. This may be fulfilled by the novel amiloride-sensitive $Na^+/H^+$ antiporter in the granule membrane, the assay of which has been described in this thesis using a number of different techniques.

The antiporter appears to be reversible and non-electrogenic, with a stoichiometry of 1:1. Using an indirect ACMA fluorescence quenching assay, the apparent $K_m$ for $Na^+$ is 4.7mM. $Li^+$ is an alternative weak substrate that can be transported with a similar $K_m$ to $Na^+$, although $K^+$ cannot replace $Na^+$. However, a direct assay using $^{22}Na^+$ suggested a higher $K_m$, closer to 20mM; $V_{max}$ values determined from $Na^+$ transport rates in pH-jump experiments in the absence of ATP were of the order of 150-200nmol.min$^{-1}$.mg$^{-1}$ protein. The antiporter is also competitively inhibited by the diuretic drug amiloride with an apparent $K_i$ of 0.26mM, although the more specific and potent analogue, ethylisopropylamiloride (EIPA) did not appear to be inhibitory.

$Na^+$-jump experiments with "ghosts" lead to acidification of the "ghost" lumen. They suggest that the antiporter is of sufficient capacity to equilibrate $Na^+$ and $H^+$ gradients across the membrane, so that the granules could have a $Na^+$ gradient as well as a $H^+$ gradient that may be used as an energy source. $Na^+$ inside "ghosts" can be used to drive the uptake of external $Ca^{2+}$ by $Ca^{2+}/Na^+$ exchange, with an apparent $K_m$ for $Ca^{2+}$ of about 1-2 μM and $V_{max}$ 50-120nmol.min$^{-1}$.mg$^{-1}$ (Fig. 5.4, page 142).

In contrast to $H^+$ entry, $Na^+$ accumulation by the "ghosts" in response to a pH jump or ATP hydrolysis produced plateau values of $Na^+$ that were well below the values predicted for a $Na^+/H^+$ antiporter of 1:1 stoichiometry. In the pH-jump experiments in the absence of ATP, the transmembrane $H^+$ gradient was dissipated very quickly, resulting in a reduced driving force for $Na^+$ uptake (Fig. 4.4, page 113); however, this explanation cannot account for the low amounts of $Na^+$ that are accumulated when the pH gradient across the membrane is being generated and maintained as a result of continuous ATP hydrolysis by the $H^+$-translocating ATPase (Fig. 4.2, page 105).

Under conditions designed to mimic the environment of a chromaffin granule in an intact cell, (ie. a transmembrane pH gradient of about 1.7pH units (acidic inside) and a large internal $Ca^{2+}$ binding capacity), addition of extravesicular $Na^+$ results in an increased uptake of $Ca^{2+}$ into the matrix to values that are similar to the free $Ca^{2+}$ concentrations found in the intact granule matrix (about 5μM) (Table 6.2, page 168). However, from the $^{22}Na^+$ uptake measurements, it is not clear whether the low amount of $Na^+$ that is accumulated within the matrix is sufficient to drive subsequent $Ca^{2+}$ uptake by $Ca^{2+}/Na^+$ exchange (Fig. 7.1). It is of course possible that the latter mechanism may not operate under these conditions.

## *7.2 Physiological Role of $Na^+$ and $Ca^{2+}$ in Cation Homeostasis in the Chromaffin Cell*

A knowledge of the level of intragranular $Na^+$ and its regulation is obviously of great importance for a proper understanding of the bioenergetic processes involving this and other ions. At the moment, the subcellular distribution of monovalent cations such as $Na^+$ and $K^+$ is not known, although a recent electron probe microanalysis study (Ornberg *et al.*, 1988) has suggested that, *in situ* , the chromaffin granule matrix is free of $Na^+$; in the case of isolated granules the $Na^+$ and

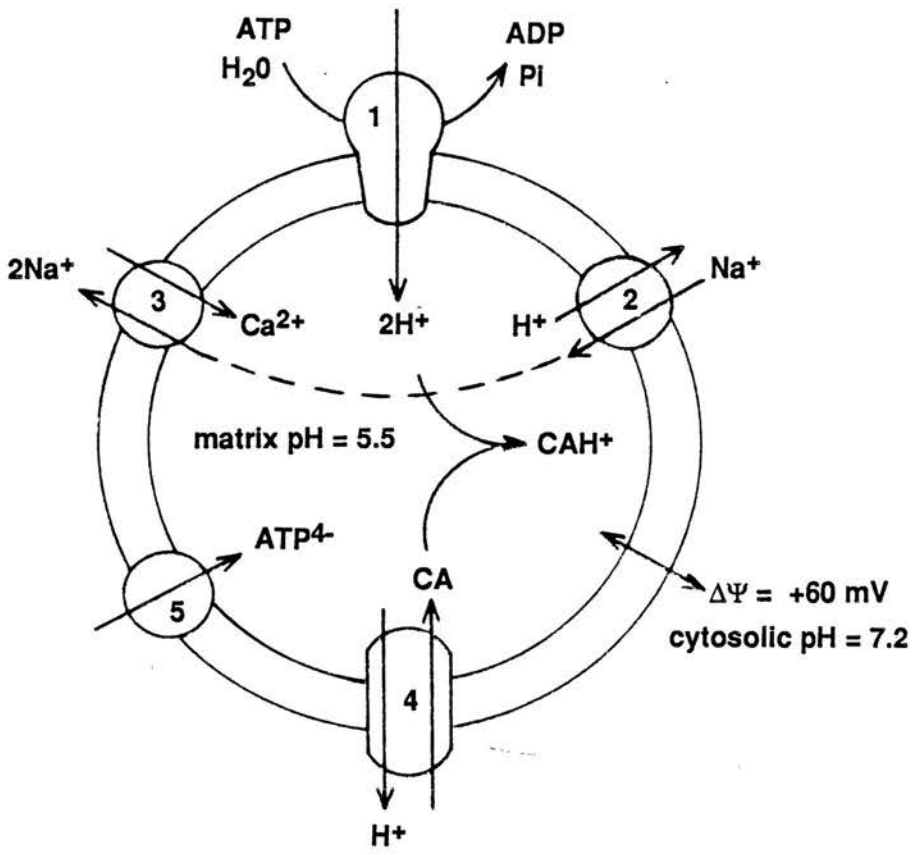



***Fig. 7.1 Interaction of $H^+$, $Ca^{2+}$, and $Na^+$ gradients in chromaffin granules.***

ATP-dependent generation of a transmembrane pH gradient by the $H^+$-ATPase **(1)** leads to the accumulation of $Na^+$ into the granule matrix, via the $Na^+/H^+$ antiporter **(2)**. Internal $Na^+$ can exchange with extravesicular $Ca^{2+}$ via the $Ca^{2+}/Na^+$ exchanger **(3)**. The other proteins shown are the catecholamine transporter **(4)** and the adenine nucleotide carrier **(5)**. $CAH^+$ is protonated catecholamine.

$K^+$ contents reflect the ionic composition of the medium used for isolation, with the lowest values being obtained in granules that are isolated in $Na^+$ and $K^+$-free buffered sucrose solutions (Chapter 6; see also Ornberg *et al.*, 1988).

The physiological role of the $Na^+/H^+$ exchanger is therefore still uncertain: one possibility is that it is involved in intracellular pH regulation (the primary role attributed to the equivalent plasma membrane carrier), in view of the large number of granules and their high surface area inside each chromaffin cell. The key question to be answered, however, is why the transmembrane $Na^+$ gradient fails to come into equilibrium with the $H^+$ gradient. One possible explanation is that there is another, and as yet unidentified, pathway of $Na^+$ transport across the membrane, in addition to the $Na^+/H^+$ and $Na^+/Ca^{2+}$ antiporters. This might explain the observation that $Na^+$ is not concentrated in the granule matrix *in vivo* . In the intact cell, $Na^+/Ca^{2+}$ exchange would therefore not operate because of the lack of an outwardly-directed transmembrane $Na^+$ gradient.

*In situ*, mitochondria from a number of sources appear to contain only 1-2 nmoles of $Ca^{2+}$ per mg of protein, as determined from recent electron probe X-ray microanalysis studies (Somlyo *et al.*, 1986), and contribute very little to cytosolic $Ca^{2+}$ regulation under normal conditions. The main function of intramitochondrial $Ca^{2+}$ is to regulate matrix dehydrogenases that occupy key sites in oxidative metabolism (McCormack & Denton, 1986). Chromaffin granules, on the other hand, contain much higher amounts of stored $Ca^{2+}$ (80-100 nmoles per mg protein from atomic absorption spectroscopic measurements; 35 nmoles per mg protein from electron probe microanalysis) although most of this is bound to acidic matrix proteins (chromogranins) and other internal binding sites. It seems likely that, *in vivo*, the granules do not constitute a large mobilisable $Ca^{2+}$ reserve; therefore, in common with mitochondria, the size of the pool of $Ca^{2+}$ that granules can contribute to the cytosol is probably very limited.

In a study of $Ca^{2+}$ homeostasis in permeabilised chromaffin cells, Kao (1988) estimated that only about 10% of the $Ca^{2+}$ that enters the cytosol following

acetylcholine-induced depolarisation of the plasma membrane would be subsequently sequestered in intracellular organelles; and that most of this $Ca^{2+}$ is handled by $Ca^{2+}$ transporting mechanisms in endoplasmic reticulum and mitochondrial membranes, in a largely ATP-dependent manner. Presumably, the rest of the $Ca^{2+}$ is extruded from the cell via the plasma membrane $Ca^{2+}$ pump and/or the $Ca^{2+}/Na^{+}$ exchanger. Kao (1988), concluded that chromaffin granules may not play a significant role in cytosolic $Ca^{2+}$ homeostasis in permeabilised cells, though more detailed studies are required to confirm or refute this suggestion, particularly in the intact cell.

Most studies of $Ca^{2+}$ and secretory granules have focused upon mechanisms of $Ca^{2+}$ transport across the granule membrane, with the emphasis being placed on the regulation of calcium homeostasis in the secretory cell. However, because of the high concentrations of stored $Ca^{2+}$, one is also tempted to think in terms of the regulation of intragranular phenomena by this cation. Two possible roles are the maintainance of granule stability and the processing of matrix proteins and peptides, and I will briefly consider each of these.

## *7.3 Role of Divalent and Monovalent Ions in the Storage of Granule Matrix Constituents*

A number of early studies by Pletscher and co-workers presented evidence for the existence of high molecular weight ATP/catecholamine aggregates in chromaffin granules (Da Prada *et al.*, 1971; Pletscher *et al.*, 1973); in model solutions the formation of these aggregates required $Ca^{2+}$ and $Mg^{2+}$, and at temperatures below 21°C a pronounced phase separation occurred. Above this temperature, however, divalent cation-dependent aggregates were absent and therefore seemed unlikely to be related to the organisation of the granule matrix at 37°C. Other studies, mainly using NMR spectroscopy, have conclusively shown that metal ions do not promote catecholamine-nucleotide interaction at pH values below

7.0 (Granot & Fiat, 1977), and, furthermore, that the high-viscosity phases referred to above are not found within granules (Sen & Sharp, 1982).

Within the matrix, ATP is critical for reducing the osmotic pressure of the stored catecholamine to that of the surrounding cytosol: weak interactions between these species gives highly non-ideal solutions, with activity coefficients of about 0.3; addition of salts of divalent ($Ca^{2+}$ and $Mg^{2+}$) or monovalent ($Na^{+}$ and $K^{+}$) cations has no effect on the osmotic pressure (Koppell & Westhead, 1982). The relatively high activity coefficients for $Na^{+}$ and $K^{+}$ (about 0.8) that I have measured in the granule matrix (Chapter 6) are consistent with this. It is interesting to note that ATP adjusted to pH 5.6 with $Na^{+}$ as counterion (2.8 $Na^{+}$ per ATP; ATP concentration 150mM, total ion concentration 570mM) has an osmotic pressure of 0.28 osM. It has been postulated that, during granule biogenesis, ATP (an anion) is stored first, at high concentrations, with the osmotic pressure reduced in this way, by ion pairing (Koppell & Westhead, 1982). Subsequent accumulation of catecholamines to their normal level could be achieved by displacement of other cations. At the moment, the chromaffin granule matrix is too complex for precise modeling, but it is clear that high molecular weight complexes are absent with weak catecholamine-ATP interactions sufficient for maintainance of osmotic stability (Sen & Sharp, 1982).

## *7.4 Is $Ca^{2+}$ Required for Processing of Proteins and Peptides Within the Chromaffin Granule Matrix?*

In secretory granules such as rat insulinoma granules, $Ca^{2+}$ and $Mg^{2+}$ are present at exceedingly high concentrations (120mM and 72mM, respectively; Hutton *et al.*, 1983); some exciting recent experiments have shown that $Ca^{2+}$, at least, is required for the processing of proinsulin and subsequent packaging of the mature insulin. It is likely that mature chromogranins and enkephalins are generated by processing of precursor proteins within the matrix of chromaffin granules.

Insulin granules contain two distinct $Ca^{2+}$-dependent acidic endoproteases (types I and II) which cleave proinsulin on the C-terminal side of specific arginine residues at junctions between the peptide chains that make up the insulin molecule (Fig. 7.2). Type I endoprotease (which cleaves on the C-terminal side of the B-chain/C-peptide junction) is half-maximally activated by 100μM $Ca^{2+}$, whereas type II endoprotease (which cleaves preferentially on the C-terminal side of the C-peptide/A-chain junction) requires millimolar $Ca^{2+}$ ($K_{0.5}$ = 2.5mM). Endoprotease activity is maximal at pH 5.5 although type II activity is displayed over a broader range of pH than that of type I; at pH 7.5 for example, type II activity was about 30-40% of that at pH 5.5, although type I activity was negligible. It has been suggested that these proteolytic activities would be maximal under the ionic conditions that exist in the insulin granule matrix (pH 5.5 and free $Ca^{2+}$ concentration 1-10mM) (Davidson *et al.*, 1988)

From pulse-chase experiments, Davidson *et al.,* (1988) have proposed that the activation of endoprotease I activity, and consequent processing of proinsulin at the B-chain/C-peptide junction, occurs as a result of acidification of the granule interior and accumulation of large amounts of $Ca^{2+}$. This would be brought about because of the insertion or activation of the insulin granule $H^{+}$-ATPase and $Ca^{2+}$ transport proteins into the membrane.

In the Golgi apparatus, where the pH is close to neutral (Orci *et al.*, 1987) and $Ca^{2+}$ is present at a lower concentration (Herman *et al.,* 1973) only type II activity would be significant and only partial processing of proinsulin would occur. Indeed, Davidson *et al.,* (1988) estimate that only about 2.5% of the proinsulin is converted to mature insulin in the Golgi complex. Thus, insulin production appears to be largely restricted to the mature secretory granule, final processing being dependent on the high matrix $Ca^{2+}$ concentration and the low matrix pH.

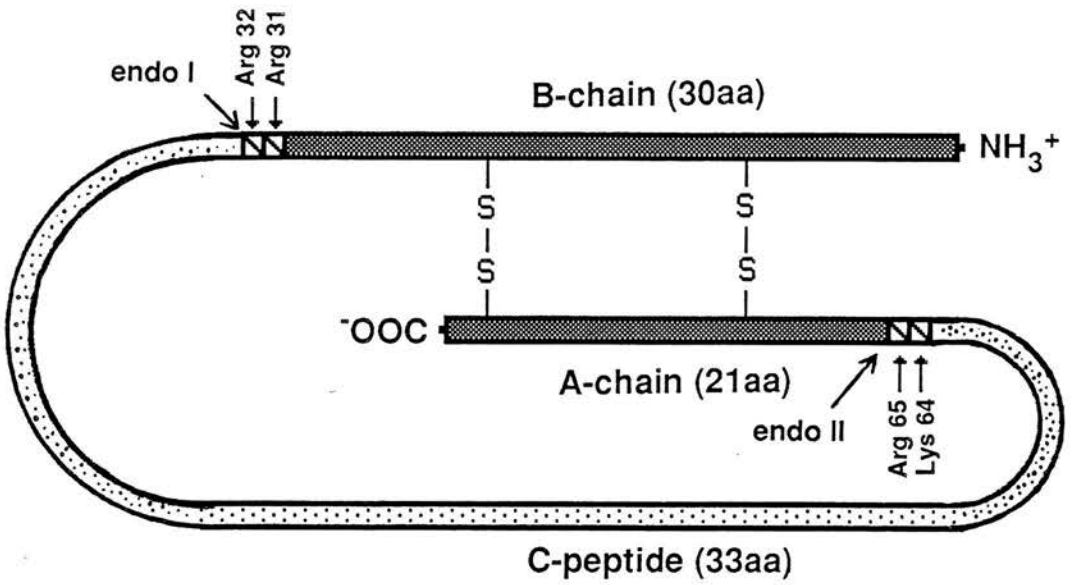



***Fig. 7.2 Processing of proinsulin in the pancreas.***

Proinsulin is cleaved by endoproteases I and II at pairs of basic amino acids in a $Ca^{2+}$- and pH-dependent manner (for details see the text). Mature insulin is then generated in secretory granules following exopeptidase-catalysed removal of these amino acid pairs.

Chromogranin A is the most abundant protein in the chromaffin granule matrix. Like insulin it is derived from a proprotein, and pulse chase experiments (Falkensammer *et al.*, 1985) have shown that during biosynthesis it becomes slightly larger and more acidic (as a result of glycosylation in the Golgi). Proteolytic processing of chromogranin A, which yields at least nine chromogranin A-like peptides, is very slow, however; in mature granules chromogranin A accounts for about 50% of the total matrix protein (Winkler *et al.*, 1986). Analysis of the primary sequence of chromogranin A reveals eight pairs of basic amino acids, the majority of which are in the C-terminal region, and which may serve as recognition signals for endoproteolytic processing to generate the smaller biologically active peptides (Grimes *et al.*, 1987).

The biological function of chromogranin A, despite many years of investigation, is still not understood. It is a $Ca^{2+}$ binding protein (Reiffen and Gratzl, 1986) and shares homology with several other $Ca^{2+}$ binding proteins such as S-100β, and parvalbumin (Grimes *et al.*, 1987). It has also been postulated to be a sorting element involved in segregation of proteins into the secretory pathway (Benedum *et al.*, 1986). It seems more likely, however, that biological activity resides in peptides derived as a result of proteolysis, as Simon *et al.*, (1988) have shown that some fragments of chromogranin A, but not the intact molecule, are inhibitory to the nicotine-evoked secretory response of cultured chromaffin cells. This is particularly interesting in view of the homology that exists between residues 243-296 of bovine chromogranin A and the sequence of porcine pancreastatin (Eiden, 1987), an inhibitor of secretion of insulin and somatostatin from the pancreas.

In insulin secretory granules, a 21kDa secreted protein of low abundance (called ß-granin) is almost indistinguishable from the N-terminus of chromogranin A (Hutton *et al.*, 1985). ß-granin in pancreatic cells is initially synthesised as a 100 kDa precursor, which is processed by a $Ca^{2+}$-dependent protease to yield ß-granin in mature secretory granules. Interestingly, the $Ca^{2+}$- and pH-dependence of

the processing activity are the same as those for insulin production (Hutton *et al.*, 1987), so that the same $Ca^{2+}$-dependent proteases are assumed to be involved.

The highly conserved sequences of the N-terminus of rat pancreatic ß-granin and bovine chromogranin A are suggestive of a common physiological function; it would clearly be of great interest to know whether the processing of chromogranin A in chromaffin granules to yield smaller peptides has a similar pH- and $Ca^{2+}$-dependence to that of proinsulin, and whether either or both of the insulinoma proteases can be identified in chromaffin granules. It would obviously be interesting to investigate the effect on processing of disrupting the chemiosmotic gradients across the granule membrane.

Like chromogranin A and its related peptides, free enkephalins in chromaffin granules are derived from a proenkephalin precursor by proteolysis. The major products are 18.2, 8.6 and 5.3 kDa peptides, which, in fact, constitute only about 2% of the total soluble proteins (Fleminger *et al.*, 1983; Patey *et al.*, 1984). A number of minor peptides (10% of the total enkephalin pool) are also derived from proenkephalin. In chromaffin granules a number of trypsin-like enzymes with pH optima 5.0 - 5.7 split enkephalin-containing peptides at the carboxy-terminal side of pairs of basic amino acids; the latter are then removed by carboxypeptidase B-like exopeptidases to yield free enkephalins which are co-released with matrix proteins and catecholamines (Hook & Eiden, 1984, 1985). However, unlike the processing of proinsulin and ß-granin in the pancreas, it is not known whether the formation of free enkephalins from precursor peptides in chromaffin granules is a $Ca^{2+}$-dependent process.

### *7.5 Identification of Cation-translocating Proteins*

An understanding of the regulation of cation homeostasis will undoubtedly be advanced if more is known about the molecular properties of the transport proteins involved. However, even in well-studied membranes such as the inner mitochondrial

membrane and plasma membranes of excitable tissues (for example heart and brain), a major problem facing experimenters is the noticeable lack of suitable drugs that can interact with cation-exchanging proteins. This has hampered purification and identificaton of the $Ca^{2+}/Na^{+}$ exchanger from cardiac sarcolemma, where the exchange activity is large and is postulated to play a key role in mediating $Ca^{2+}$ fluxes across the membrane, and hence contractile function (Reeves, 1985). So far, the functonal $Ca^{2+}/Na^{+}$ exchanger, which accounts for 0.1-0.2% of the membranes protein, has been reconstuted into liposomes following detergent solubilisation of plasma membrane (Cheon & Reeves, 1988); however, SDS gel electrophoresis reveals the presence of many other plasma membrane proteins and it is not yet possible to make a definite identification of the exchange carrier.

Recently, a number of terminal guanidino-substituted analogues of amiloride, such as benzamil and dichlorobenzamil, have proved useful in studies of sarcolemmal $Ca^{2+}/Na^{+}$exchange (Slaughter *et al.*, 1988). The kinetics of inhibition are rather complex, however, indicating an interaction of the inhibitors with at least two different $Na^{+}$ binding sites. Moreover, these amiloride derivatives interfere with other cardiac membrane ion-transporting systems, such as voltage-dependent $Ca^{2+}$ channels (Bielfeld *et al.*, 1986); their use in investigating the physiological role of $Ca^{2+}/Na^{+}$ exchange should, therefore, be regarded with caution. More selective inhibitors which may aid purification and identification of the exchanger, as well as clarifying its physiological function, need to be found.

Similar appoaches have been adopted towards identification of the $Na^{+}/H^{+}$ antiporter that is present in most eukaryotic cell plasma membranes. The $Na^{+}/H^{+}$ exchanger in rabbit renal brush border membranes has been solubilised and reconstituted into phospholipid vesicles, as a first step towards eventual isolation (Weinman *et al.*, 1988); in another study, $^{14}C$-labelled dicyclohexylcarbodiimide (DCCD) was used as an affinity label of the $Na^{+}/H^{+}$ exchanger in renal brush border membranes of the rat (Friedrich *et al.*, 1986). [$^{14}C$]DCCD was incorporated into polypeptides of apparent molecular weight 88, 65 and 51 kDa; labelling of the 65

kDa component was amiloride-sensitive. Similarly, the photoreactive amiloride derivative 5-ethylisopropyl-6-bromoamiloride (Br-EIPA) was mainly incorporated into the 65 kDa polypeptide, and the labelling was partly reduced by inclusion of the natural substrate $Na^+$ in the incubation mixture. It seems likely, therefore, that the 65 kDa polypeptide is involved in the $Na^+/H^+$ exchange reaction (Friedrich *et al.*, 1986)

In chromaffin granule membranes, only a few proteins of known function and composition have been identified, and only the most abundant have been purified to homogeneity. None of the carriers involved in the transport of catecholamines, nucleotides, calcium or sodium have been identified as specific proteins.

One of the main questions to be answered is whether or not the $Na^+/H^+$ antiporter in chromaffin granules is identical with the similar exchangers of plasma membranes. The kinetics and pharmacological profile of the antiporter need to be investigated more thoroughly. One approach would be the use of several different analogues of amiloride. The unexpected and important finding that ethylisopropyl amiloride (EIPA) appeared not to be inhibitory for the granule $Na^+/H^+$ antiporter requires detailed investigation. However, this may not be altogether straightforward, since EIPA appears to act as an uncoupler (proton ionophore), causing dissipation of the transmembrane proton gradient (see Chapter 4). A number of other inhibitors of the plasma membrane exchanger of broader specificity could also be used, including sulphydryl reagents such as N-ethylmaleimide (Pierce *et al.*, 1986), and DCCD (Freidrich *et al.*, 1986).

Recently, Barbry *et al.*, (1987) have successfuly solubilised, purified and identified the subunit structure of the aldosterone-regulated $Na^+$ channel of pig kidney distal tubules using the potent amiloride derivative [$^3$H]phenamil. Chromaffin granule membranes can be obtained in relative abundance and purity and would, therefore, be a good starting point for similar studies. Fractionation of chromaffin granule membranes by phase separation after treatment with the detergent Triton X-114 (Pryde & Phillips, 1986), demonstrates that most of the

protein components have a characteristic distribution which can be exploited for subsequent purification. Used in combination with specific labelling reagents, this procedure might simplify the search for the cation-translocating proteins.

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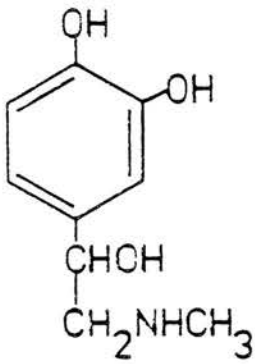
***Appendix.*** ***Formulae of some of the compounds mentioned in the text***



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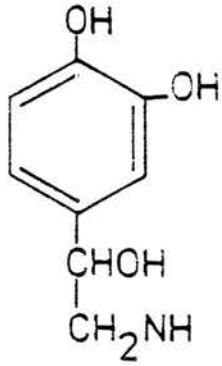

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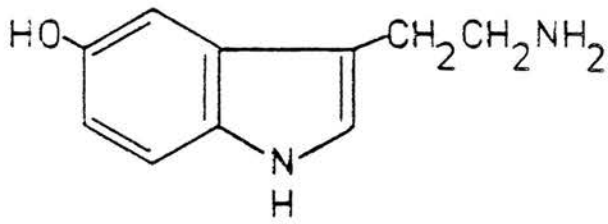

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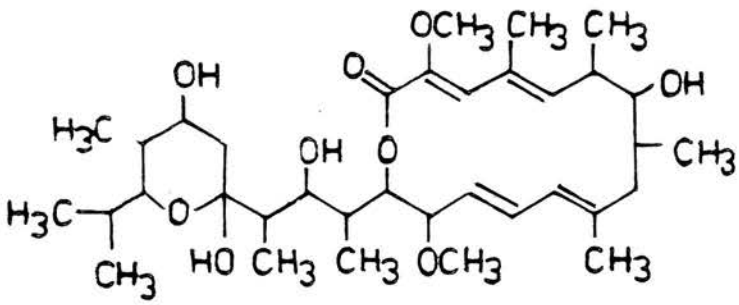

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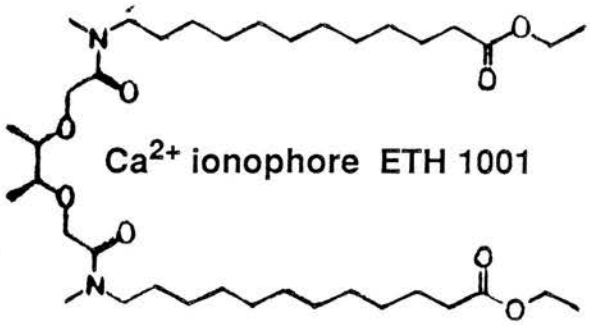

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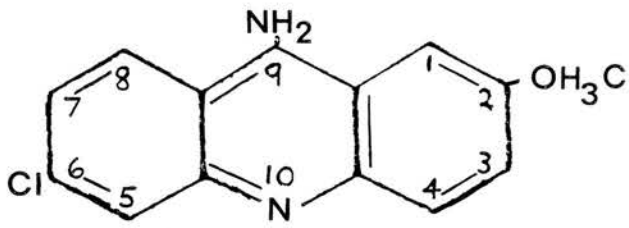

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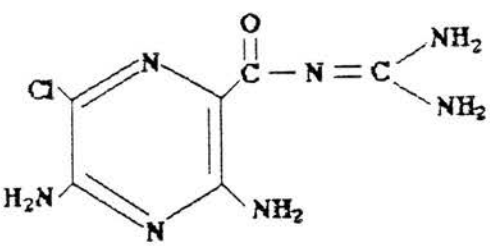

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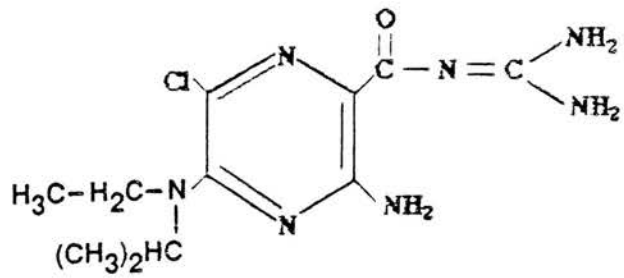

Ethylisopropyl amiloride (EIPA)

Biochem. J. (1989) 257, 499–507 (Printed in Great Britain)



# A sodium/proton antiporter in chromaffin-granule membranes

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Chromaffin granules, the secretory vesicles of the adrenal medulla, have a $Na^+/H^+$ exchange activity in their membranes which brings their proton gradient into equilibrium with a $Na^+$ gradient. This explains why $Na^+$ is mildly inhibitory to amine transport (which is driven by the $H^+$ gradient). The activity can be demonstrated by using accumulation of $^{22}Na^+$ in response to a pH gradient that is either imposed by diluting membrane 'ghosts' into alkaline media, or generated by ATP hydrolysis. It can also be monitored indirectly by fluorescence measurements in which the pH inside 'ghosts' is monitored by quenching of a fluorescent weak base. This method has been used to monitor $Na^+$ entry into acid-loaded 'ghosts' or $H^+$ entry into $Na^+$-loaded 'ghosts'. $Na^+$-jump experiments also lead to acidification of the interior, as demonstrated by methylamine accumulation. The exchanger appears to be reversible and non-electrogenic, with a stoichiometry of 1:1. Using an indirect assay we measured an apparent $K_m$ for $Na^+$ of 4.7 mM, and a $K_i$ for amiloride, a competitive inhibitor, of 0.26 mM. Direct assays using $^{22}Na^+$ suggested a higher $K_m$. Ethylisopropylamiloride was not inhibitory.

## INTRODUCTION

Chromaffin granules, the secretory vesicles of the adrenal medulla, contain high concentrations of catecholamines and nucleotides as well as various proteins and biologically active peptides. Like many other secretory vesicles, they have an acidic matrix; in isolated granules this appears to be buffered at about pH 5.7 (Johnson & Scarpa, 1976). Similar or slightly lower values are probably found in intact tissues (Holz *et al.*, 1983; Bevington *et al.*, 1984). This acidic milieu is generated and maintained by a V-type (Mellman *et al.*, 1986; Pederson & Carafoli, 1987) proton-translocating ATPase which utilizes cytosolic ATP for inwardly directed electrogenic proton translocation. The resulting proton and potential gradients are used as an energy source for catecholamine transport (see review by Njus *et al.*, 1987*a*).

In this paper we show that chromaffin-granule membranes contain a reversible $Na^+$/proton antiporter. We suggest that this is of sufficient capacity to equilibrate $Na^+$ and $H^+$ across the membrane, so that the granules have a $Na^+$ gradient as well as a proton gradient that may be used as an energy source. This would be consistent with the long-standing observation that $Ca^{2+}$ transport by the granules is $Na^+$-dependent, and is not linked directly to protons (Phillips, 1981; Krieger-Brauer & Gratzl, 1982).

## MATERIALS AND METHODS

### Materials

Bovine adrenal glands were placed on ice within about 30 min of slaughter. They were transported to the laboratory and chromaffin granules were prepared within the next 2 h (Phillips, 1974*a*). All radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K. 9-Amino-6-chloro-2-methoxyacridine (ACMA) was a gift from Dr. R. Kraayenhof (Vrije University, Amsterdam, The Netherlands), and ethylisopropylamiloride was a gift from Professor F. Lang, Institut für Physiologie, University of Innsbruck, Austria. Cellulose nitrate filters (pore size 0.45 $\mu$m) were from Schleicher and Schüll, Dassel, West Germany.

Hepes and Mes were generally prepared as 1 M solutions and adjusted to the required pH with NaOH, KOH or tetramethylammonium (TMA) hydroxide. pH values are quoted for the concentration used in experiments. EGTA was also adjusted to the required pH; it was included in most media to decrease $Ca^{2+}$ movements coupled to $Na^+$.

### Methods

**Preparation of 'ghosts'.** Resealed chromaffin-granule 'ghosts' were prepared by the method of Apps *et al.* (1980): this involves lysis of fresh granules by 50-fold dilution into a lysis buffer, collection by centrifugation, purification through a sucrose/$^2H_2O$ gradient, and storage at −20 °C. Solutions were generally buffered with 10 mM-Hepes as its $K^+$ salt, pH 7.0. When 'ghosts' were loaded at pH 6.0, lysis was performed in a solution containing 10 mM-Mes and 0.1 mM-EGTA as their $K^+$ salts, pH 6.0, and solutions used in purification were also buffered with 10 mM-Mes, pH 6.0. $Na^+$-loaded 'ghosts' were prepared by lysis of granules in 10 mM-Hepes/0.1 mM-EGTA/KOH (pH 7.0) containing 25 mM-$Na_2SO_4$; solutions used in subsequent purification then also contained 25 mM-$Na_2SO_4$. Protein concentrations of 'ghost' suspensions were determined by the method of

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxyacridine; TMA, tetramethylammonium; 5HT, 5-hydroxytryptamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Bradford (1976), with bovine serum albumin as a standard.

**Transport assays**. ATP-dependent uptake of 5-hydroxy[$^{14}$C]tryptamine ([$^{14}$C]5HT) was determined by filtration of 'ghosts' as described previously (Apps *et al.*, 1980), samples being taken at 20 s intervals over 3 min for initial-rate determinations. Simultaneous accumulation of 5HT and $Na^+$ was measured by incubating 'ghosts' (0.15 mg/ml) at 30 °C in a medium containing 0.3 M-sucrose, 30 mM-Hepes/KOH, pH 7.0, 6 mM-$ATPK_2$, 3 mM-$MgSO_4$, 20 mM-KI, 100 $\mu$M-$^{22}Na_2SO_4$ (3 $\mu$Ci/ml), 50 $\mu$M-[$^3$H]5HT (1 $\mu$Ci/ml). Samples (100 $\mu$l) were removed at intervals to ice-cold 0.3 M-sucrose/30 mM-Hepes/KOH, pH 7.0, containing 10 $\mu$M-5HT (2.5 ml). $Na_2SO_4$ was added to 5 mM before filtration. Filters were washed with 2 × 2.5 ml of ice-cold 0.3 M-sucrose/10 mM-Hepes/NaOH, pH 7.0, and radioactivity was determined by scintillation counting.

For analysis on a sucrose gradient, the incubation mixture (20 min at 30 °C) was centrifuged through a small column (2 cm × 0.9 cm diam.) of Bio-Gel P6DG prepared in a Microfuge tube with a hole in the bottom. The Bio-Gel was equilibrated with cold 0.3 M-sucrose/30 mM-Hepes/KOH, pH 7.0. The 'ghosts' were then applied to a linear gradient (13 ml) of sucrose (0.3–1.3 M) in 30 mM-Hepes/KOH, pH 7.0, and centrifuged at 196000 ***g*** for 150 min at 4 °C in an SW41 rotor in a Beckman ultracentrifuge. Fractions (0.6 ml) were collected, and 0.4 ml portions were added to 2.5 ml of buffered 0.3 M-sucrose for filtration as above. Fraction densities were calculated from refractive-index measurements made on a parallel gradient. Acetylcholinesterase (EC 3.1.1.7) and cytochrome *c* oxidase (EC 1.9.3.1) activities were measured in samples of fractions as described by Phillips (1981).

Accumulation of $^{22}Na^+$ by low-pH 'ghosts' was achieved by diluting 'ghosts' made at pH 6.0 (3–6 mg/ml) 30–40-fold into media at 25 °C at the required pH containing 0.3 M-sucrose, 20 mM-Hepes, 0.1 mM-EGTA/KOH and various concentrations of NaCl in the presence of $^{22}$NaCl at 4 $\mu$Ci/ml. Samples (100 $\mu$l) were removed for filtration as above. In some experiments, a drop (8 $\mu$l) of 'ghost' suspension was placed on the side of a plastic tube (4 ml, flat-base polypropylene; Sarstedt, Leicester, U.K.) containing 200 $\mu$l of incubation medium. Uptake was initiated by mixing the pre-warmed (25 °C) contents on a vortex mixer, and was quenched by rapid addition of 3.5 ml of ice-cold 0.3 M-sucrose/10 mM-Hepes/KOH, pH 7.0, containing 5 mM-$Na_2SO_4$.

Acidification of $Na^+$-loaded 'ghosts' was measured by dilution of 'ghosts' into a $Na^+$-free medium containing 0.3 M-sucrose, 30 mM-Hepes/TMA, pH 7.3, 0.1 mM-EGTA, 50 mM-choline chloride, with 2.5 $\mu$M-[$^{14}$C]-methylamine (0.15 $\mu$Ci/ml). The final $Na^+$ concentration in the medium was 1.7 mM. The logarithm of the methylamine concentration ratio (concentration inside/concentration outside) is equal to the magnitude of the pH gradient (outside minus inside).

$Na^+/Na^+$ exchange was measured by incubating 'ghosts' prepared in 0.3 M-sucrose/10 mM-Hepes/NaOH, pH 7.0 (4 mM-$Na^+$), at 37 °C in the same medium containing 0.1 mM-EGTA/NaOH for up to 2 h. $^{22}$NaCl (1.5 $\mu$Ci/ml) was added at intervals, and accumulation was measured by filtration. For estimating the accumulated concentrations of $Na^+$ and amines, we used a value of 3.6 $\mu$l/mg for the internal volume of the 'ghosts' (Phillips & Allison, 1978) when using media of low ionic strength, but a value of 3 $\mu$l/mg when media were supplemented with salt solutions.

In general, kinetic results presented in this paper are typical experiments from a series performed on several preparations of 'ghosts'. The filtration assays gave results reproducible within ±3% (S.E.M. for 5HT or methylamine), or ±15% for $^{22}$Na, in which the ratio of accumulated d.p.m. to background is much lower.

**Fluorescence experiments**. Assays were performed in media (25 °C; final volume 0.5 ml) containing 0.3 M-sucrose, 10 mM-Hepes and 0.1 mM-EGTA/KOH, pH 7.0. NaCl or LiCl was added, with choline chloride to make up the concentration to 60 mM. ACMA was used as a stock solution (50 $\mu$g/ml) in ethanol, and was added to 0.75 $\mu$M final concn. 'Ghosts' (internal pH 6.0) were used at 0.10–0.15 mg/ml. Excitation (slit-width 5 nm) was at 420 nm, and emission was measured (slit-width 10 nm) at 525 nm in a Perkin–Elmer 3000 fluorimeter. Amiloride was added from a 10 mM stock in water, and ethylisopropylamiloride from a 5 mM stock in ethanol.

All experiments were performed on several preparations of 'ghosts'. Representative traces are presented in this paper.

Alternatively, $Na^+$-loaded 'ghosts' were added to media containing 0.3 M-sucrose, 30 mM-Hepes, 12 mM-TMA, 50 mM-choline chloride and 0.75 $\mu$M-ACMA, pH 7.30, and quenching of ACMA fluorescence was monitored. Responses were calibrated (Warnock *et al.*, 1982) by diluting 'ghosts' loaded with Mes at pH 6.0 into media containing 0.3 M-sucrose, 30 mM-Hepes, 0.1 mM-EGTA at various pH values, adjusted with TMA hydroxide. Results were plotted according to eqn. (1):

$$\log\left(\frac{A_i}{A_o}\right) = \Delta pH + \log\left(\frac{v_i}{v_o}\right) \quad (1)$$

where $A_i$ and $A_o$ are the total amounts of ACMA inside and outside the 'ghosts', $\Delta pH$ is the difference in pH (outside minus inside) across the 'ghost' membrane, and $v_i$ and $v_o$ are the volumes of the intra- and extra-vesicular spaces. $A_i$ is measured by the percentage of fluorescence quenched and $A_o$ by the initial fluorescence (100% value) minus the percentage quenched.

Measurements of percentage quenching were then used for determination of $\Delta pH$. The validity of this approach was checked by adding 2 M-HCl to cuvettes in an amount that eliminated quenching, and then measuring the pH of the medium: fair agreement was found between the two methods.

## RESULTS

### $Na^+$ inhibition of 5-HT accumulation

Experiments on catecholamine transport by chromaffin granules generally utilize resealed membrane 'ghosts', rather than intact granules, which tend to lyse or to leak their constituents during incubations (Phillips, 1974*a*). Such 'ghosts' have commonly been prepared in media buffered with Hepes in the form of its $Na^+$ salt, but we have recently found that $Na^+$ is mildly inhibitory in these transport assays.

5HT is an excellent substrate for the amine transporter, with a $K_m$ of 5–8 $\mu$M (Phillips, 1974*b*; Carty *et al.*, 1985).

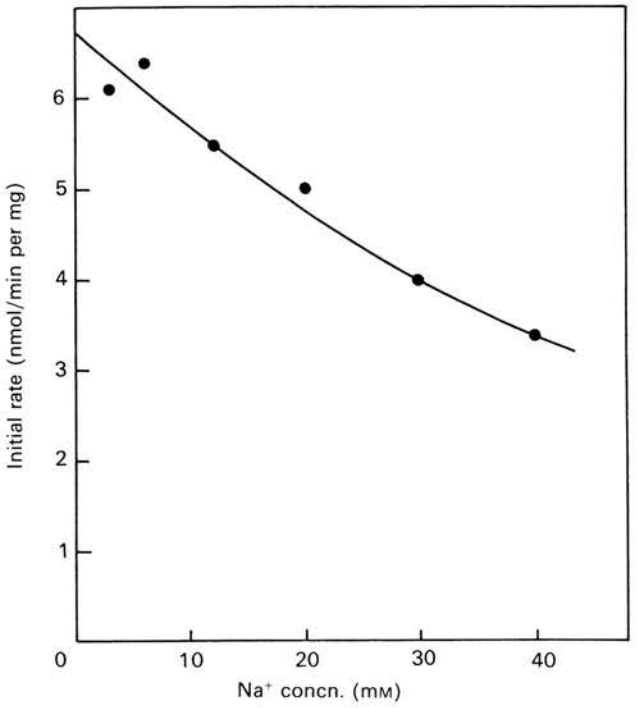


**Fig. 1. $Na^+$ inhibition of 5HT accumulation by 'ghosts'**

Chromaffin-granule 'ghosts' (0.11 mg/ml) were incubated with 50 $\mu$M-[$^{14}$C]5HT and MgATP in media supplemented with various concentrations of $Na_2SO_4$; osmolarity was maintained constant by addition of $K_2SO_4$. Initial rates of accumulation of 5HT at 30 °C were measured at each $Na^+$ concentration by filtering samples at intervals of 20 s.

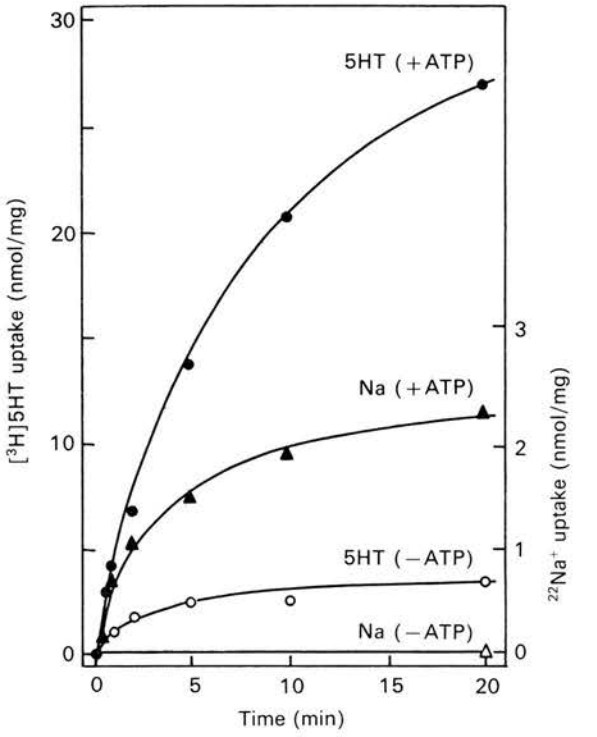


**Fig. 2. Accumulation of $Na^+$ and 5HT by 'ghosts'**

Chromaffin-granule 'ghosts' (0.15 mg/ml) were incubated at 30 °C in a standard medium supplemented with 20 mM-KI, 50 $\mu$M-[$^3$H]5HT (●, ○) and 100 $\mu$M-$^{22}Na_2SO_4$ (▲, △). Incubations were performed in the presence (●, ▲) or absence (○, △) of MgATP.

We have re-investigated the initial rates of its transport into resealed 'ghosts' by using various concentrations of $Na^+$ in the incubation media, with $K^+$ present to maintain the ionic strength (Fig. 1). $Na^+$ proves to be weakly inhibitory. The amine transport is linked to the protonmotive force generated across the 'ghost' membrane by ATP hydrolysis by a process that involves exchange of amine for a proton (Njus *et al.*, 1987*a*). The activity of the proton-translocating ATPase, however, is unaffected by $Na^+$ concentrations below 50 mM. These observations therefore suggested that $Na^+$ might have a weak uncoupling role in this system, and we show below that this results from an initial decrease in the proton gradient by exchange of $H^+$ with $Na^+$.

### ATP-driven $Na^+$ transport

The establishment of a transmembrane pH gradient in the 'ghosts' can be used to drive accumulation of $^{22}Na^+$. The $Na^+/H^+$ antiporter has a relatively high $K_m$ for $Na^+$, so that such experiments are difficult, the accumulation of the radioactive $^{22}Na^+$ being masked by non-radioactive $Na^+$; the experiments are thus performed at $Na^+$ concentrations that are well below $K_m$. In the experiment shown in Fig. 2, 'ghosts' were incubated with MgATP, 20 mM-KI (to increase $\Delta$pH at the expense of $\Delta\psi$), 50 $\mu$M-[$^3$H]5HT and 100 $\mu$M-$^{22}Na_2SO_4$, so that the use of the proton gradient for amine and $Na^+$ transport could be examined simultaneously.

Fig. 2 shows that $Na^+$ equilibrates across the membrane. Osmotic lysis released both 5HT and $Na^+$ (results not shown), and the uptake of both is dependent on MgATP. After about 20 min the plateau value of accumulated $Na^+$ (about 2.5 nmol/mg) corresponds to an internal concentration of approx. 0.8 mM, assuming an internal volume of about 3 $\mu$l/mg of protein, slightly less than the value measured by Phillips & Allison (1978) at lower osmolarity. This is equivalent to a concentration gradient of about 8-fold across the membrane. In contrast, 5HT uptake is far more extensive and proceeds

**Table 1. Effects of inhibitors on $Na^+$ and 5HT accumulation by 'ghosts'**

'Ghosts' were incubated with [$^3$H]5HT and $^{22}Na^+$ as described in Fig. 2. Values given are related to control values (in the presence of ethanol if appropriate) after 20 min incubation, and are means ± S.E.M. ($n$ = 3).

| Addition to medium | Accumulation (% of control value) 5HT | $Na^+$ |
|---|---|---|
| Ethanol (0.5%) | 111±2.6 | 120±9.0 |
| $(NH_4)_2SO_4$ (10 mM) | 1±0.7 | 31±5.5 |
| FCCP (10 $\mu$M) | 19±1.0 | 62±3.2 |
| Ouabain (1 mM) | 102±5.9 | 106±14.2 |
| Sodium orthovanadate (10 $\mu$M) | 125±4.7 | 109±1.6 |
| *N*-Ethylmaleimide (30 $\mu$M) | 32±2.7 | 30±4.6 |
| Reserpine (10 $\mu$M) | 10±3.0 | 90±9.2 |
| Amiloride (1 mM) | 105±4.0 | 68±12.2 |

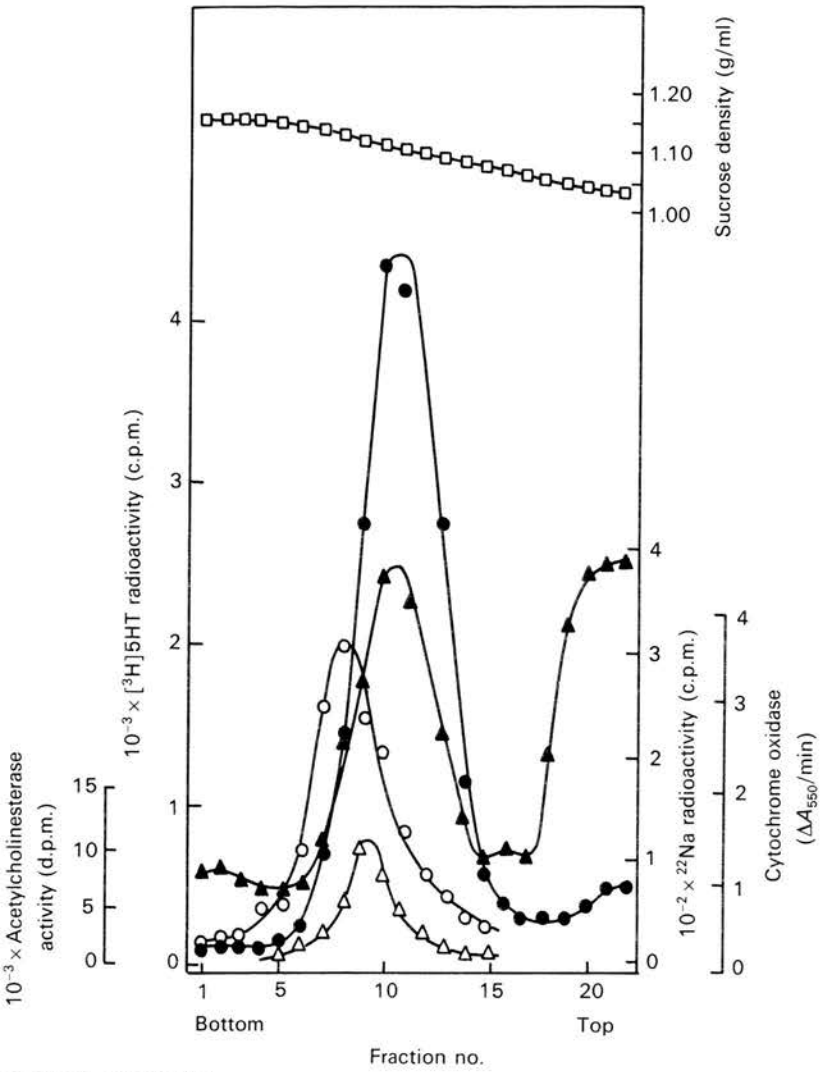


**Fig. 3. Sucrose-gradient analysis of 'ghosts'**

Chromaffin-granule 'ghosts' were incubated with MgATP, [$^3$H]5HT and $^{22}Na_2SO_4$ as described in Fig. 2. After 20 min at 30 °C they were analysed on a sucrose gradient. Portions of the fractions collected were filtered and analysed for radioactivity ([$^3$H]5HT, ●; $^{22}$Na, ▲) or were assayed for acetylcholinesterase (△) or cytochrome oxidase (○) activities. Refractive-index measurements made on a parallel gradient were used for calculation of fraction densities (□).

for longer, a concentration gradient of about 200-fold being demonstrated in Fig. 2. The high concentration gradient achieved with the amine (Phillips & Apps, 1980) results from the electrogenic exchange of two protons for each amine cation that is catalysed by the amine transporter (or, more probably, the exchange of one proton for an uncharged amine molecule; Scherman & Henry, 1981). It achieves equilibrium with the proton electrochemical gradient more slowly than the $Na^+/H^+$ exchange.

We used the assay shown in Fig. 2 to examine the effects of some inhibitors on the two transport systems (Table 1). It was too difficult to measure initial rates accurately, so that accumulation at 20 min is given in Table 1. Under these conditions, 5HT uptake was $23.2 \pm 1.72$ nmol/mg (S.E.M. for the three different 'ghost' preparations) and $Na^+$ uptake was $1.85 \pm 0.29$ nmol/mg. Transport of both 5HT and $Na^+$ was decreased by inclusion of the uncoupler FCCP, by $NH_4^+$ (which decreases the pH gradient across the membrane), and by *N*-ethylmaleimide, an inhibitor of the $H^+$-ATPase (Percy & Apps, 1986). Neither substrate was affected by ouabain or vanadate, which do not affect this ATPase (Percy *et al.*, 1985). Reserpine is a potent inhibitor of the amine transporter, but also has some detergent-like effect on the 'ghosts' (Zallakian *et al.*, 1982). Amiloride inhibits the accumulation of $Na^+$.

We also used this assay method (simultaneous incubation of 'ghosts' with [$^3$H]5HT and $^{22}Na^+$ in the presence of ATP) to show that the $Na^+$ is accumulated into the same compartment as the amine. After the incubation, we subjected the 'ghosts' to equilibrium sedimentation on a sucrose density gradient. The 'ghosts' used in these experiments are already highly purified, by a method (Apps *et al.*, 1980) that is itself based on density-gradient centrifugation, so that contaminating organelle fragments are very close in density to the 'ghosts' themselves. Contaminants can, however, be resolved from 'ghosts' on a further gradient, and the dual-label technique is used as a sensitive assay for co-equilibration.

The results shown in Fig. 3 demonstrate that $^{22}Na^+$ is

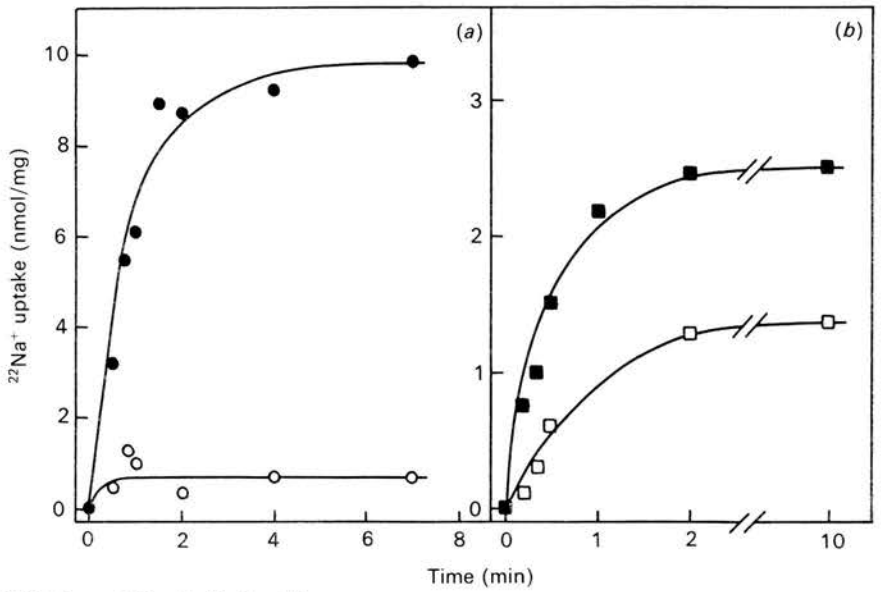


**Fig. 4. Accumulation of $Na^+$ by acid-loaded 'ghosts'**

Chromaffin-granule 'ghosts' loaded with buffer at pH 6.0 were diluted to a concentration of 0.2 mg/ml, into media at 30 °C as described under 'Methods'. These media contained (*a*) 1.0 mM-$^{22}NaCl$ (4 $\mu$Ci/ml) with final pH values of 8.0 (●) and 6.0 (○), or (*b*) 0.5 mM-$^{22}NaCl$ (4 $\mu$Ci/ml) at pH 7.0, without (■) or with 1 mM-amiloride (□).

co-sedimenting with the incorporated amine. Radioactivity at the top of the gradient (similar amounts of [$^3$H]5HT and $^{22}Na^+$) is not in particulate material, but results from some carry-over of the radioisotopes from the incubation medium (or, possibly, leakage from the 'ghosts'). Acetylcholinesterase activity is found in the matrix of intact chromaffin granules (Gratzl, 1984; Burgun *et al.*, 1985), but, when membrane 'ghosts' are used, this activity can be used as a convenient marker for plasma-membrane fragments; both it and cytochrome oxidase (a marker for mitochondria) sediment at slightly higher densities than the 'ghosts', as found in previous studies (Phillips, 1981).

### pH-jump experiments

The experiments described above suggested that $Na^+$ could enter the 'ghosts' in response to the activity of the 'ghost' membrane $H^+$-ATPase. We have made a direct demonstration that uptake is in response to the pH gradient generated by this ATPase by using 'ghosts' lysed and resealed in the presence of Mes buffered at pH 6.0, and then purified in the usual way. These acid-loaded 'ghosts' were diluted into media at higher pH that contained $^{22}Na^+$, but no ATP. There is a rapid accumulation of $^{22}Na^+$ (Fig. 4); after about 2 min this reaches a maintained plateau value that depends on the pH gradient. In the experiments shown, the initial pH gradients were 1.0 or 2.0 pH units. The internal $Na^+$ concentration found at the plateau value when $\Delta pH = 1$ was approx. 0.7 mM, in the presence of 0.5 mM-$Na^+$ in the medium, and when $\Delta pH = 2$ the plateau value was 2.7 mM with 1.0 mM-$Na^+$ externally.

We attempted to use this method to investigate the kinetics of $Na^+$ accumulation, using a pH jump from pH 6 to 7, and $Na^+$ concentrations in the range 0.5–50 mM. The shortest incubation time that it was practicable to use, however, was 10 s, and, as shown in Fig. 4(*b*), the accumulation is non-linear at this point. Estimates of the $K_m$ for $Na^+$ were of the order of 20–30 mM, however, with $V_{max.}$ values as high as 150–200 nmol/min per mg. We were unable to make more accurate measurements than this.

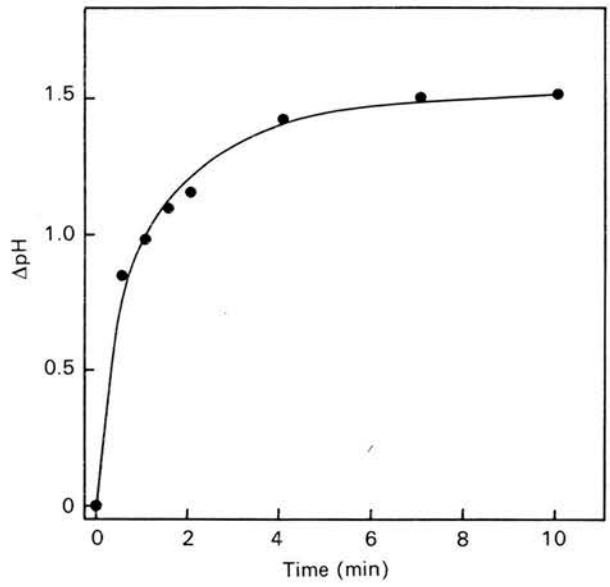


**Fig. 5. Generation of a pH gradient by $Na^+$-loaded 'ghosts'**

Chromaffin-granule 'ghosts' containing 25 mM-$Na_2SO_4$ were diluted into medium at 25 °C to give a protein concentration of 0.12 mg/ml and a final $Na^+$ concentration of 1.7 mM. 'Ghost' acidification was followed by accumulation of [$^{14}$C]methylamine and is plotted as the pH gradient ($\triangle$pH) across the membrane.

Fig. 4 also demonstrates that this $H^+$-driven $Na^+$ accumulation is sensitive to amiloride, an inhibitor of plasma-membrane $Na^+/H^+$ exchangers. This is explored further below.

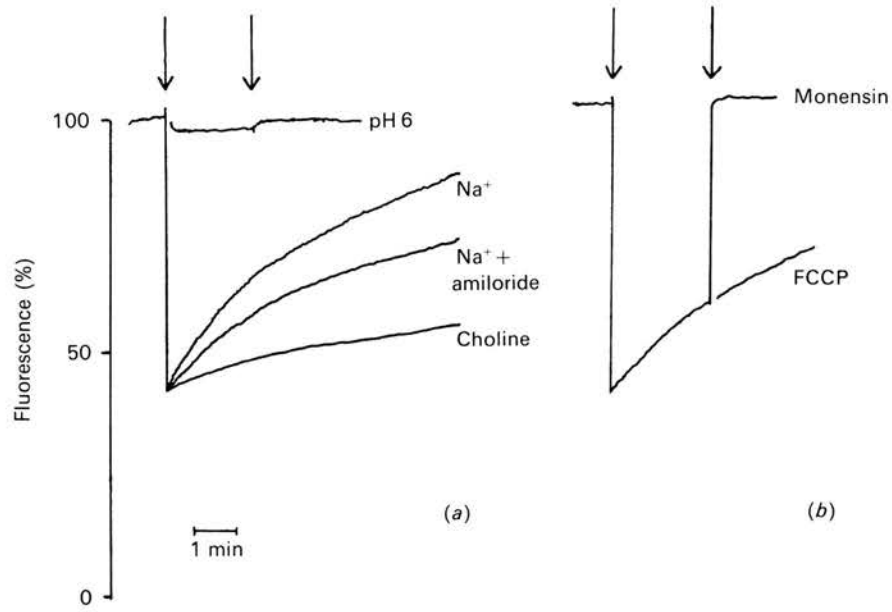


**Fig. 6. Demonstration of $Na^+$ entry into acid-loaded 'ghosts' by using ACMA fluorescence**

(*a*) 'Ghosts' loaded at pH 6.0 were diluted into medium at pH 7.0 (25 °C) containing ACMA at the times shown by the first arrow, and fluorescence was recorded. Media were supplemented with 50 mM-choline chloride, 50 mM-NaCl, or 50 mM-NaCl plus 1 mM-amiloride. In a control experiment, the medium was at pH 6.0; 50 mM-NaCl was added at the second arrow. (*b*) As (*a*), with medium containing 50 mM-NaCl; at the second arrow was added either 10 $\mu$M-monensin or 10 $\mu$M-FCCP. In all cases the final protein concentration was 0.12 mg/ml, and the time and fluorescence scales were as shown in (*a*).

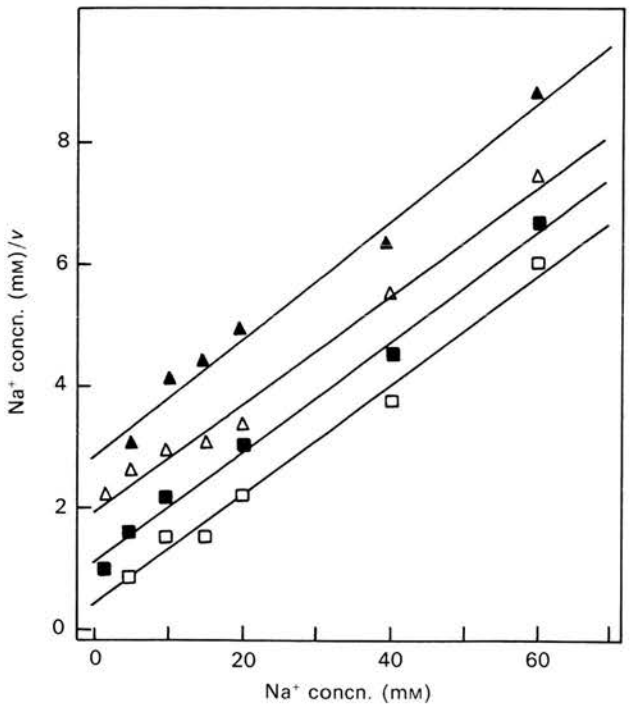


**Fig. 7. Competitive inhibition of $Na^+/H^+$ antiporter by amiloride**

$Na^+$ entry into acid-loaded 'ghosts' was assayed by ACMA fluorescence as described in Fig. 6. Media were supplemented with NaCl and choline chloride to make a total salt concentration of 60 mM. Initial rates of recovery of ACMA fluorescence ($v$, arbitrary units) were measured in the absence of amiloride (□) or in the presence of (■) 0.5 mM-, (△) 1.0 mM- or (▲) 1.5 mM-amiloride.

### $Na^+$-jump experiments

Reversibility of the exchanger was examined by diluting $Na^+$-loaded 'ghosts' into medium containing no $Na^+$ but at the same pH (7.0) as their internal pH. A trace amount of [$^{14}$C]methylamine was added in order to follow the generation of a pH gradient ($\Delta$pH) across the membrane (acid inside) in response to $Na^+$ efflux. Such a gradient is in fact established rapidly, and is maintained for a considerable time (Fig. 5). In the experiment shown, a 30-fold gradient of $Na^+$ across the membrane sustained a pH gradient of 1.5 units (a 30-fold $H^+$ concentration gradient), suggesting a stoichiometry of the exchanger of 1:1 if it is the only mechanism for linking the two gradients.

### Fluorescence assay of $Na^+/H^+$ exchange

Because of the difficulty noted above of making a kinetic analysis of the exchanger by using $^{22}$Na, in view of its high $K_m$ for Na, we used another method for direct monitoring of transmembrane proton movements in response to influx or efflux of $Na^+$. This employed the fluorescent weak base ACMA.

We first calibrated the quenching of ACMA fluorescence by adding 'ghosts' containing 10 mM-Mes, pH 6.0, to media buffered at various pH values, by using the method described by Warnock *et al.* (1982). ACMA accumulation by the 'ghosts' in response to the pH gradient leads to quenching of fluorescence, and this is a function of the magnitude of the pH gradient. We plotted this according to eqn. (1), obtaining a straight-line plot with a slope of 0.6. The deviation from the ideal value of 1.0 may be ascribed to dye binding to the membranes (Warnock *et al.*, 1982). The measured slope was found to

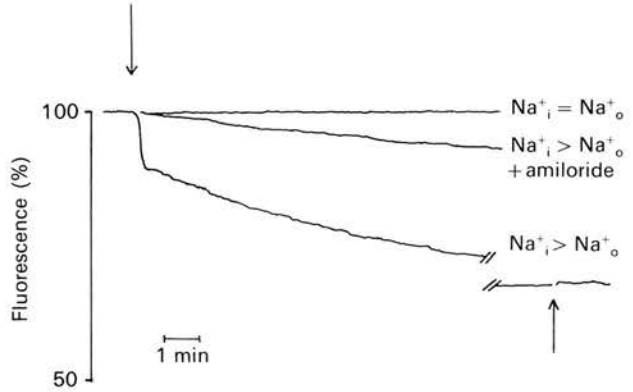


**Fig. 8. Acidification of $Na^+$-loaded 'ghosts'**

'Ghosts' loaded with 25 mM-$Na_2SO_4$ at pH 7.0 were diluted into medium (pH 7.0, 25 °C) containing ACMA at the time shown by the first arrow to give a concentration of 0.12 mg/ml, and fluorescence was monitored. In the control experiment, the medium contained 25 mM-$Na_2SO_4$ so that no $Na^+$ gradient was established. In the other experiments the final medium contained 0.85 mM-$Na_2SO_4$ ($Na^+_o = 1.7$ mM), with or without 1 mM-amiloride. At the second arrow (30 min after the start), either 10 $\mu$M-monensin or 10 $\mu$M-FCCP was added.

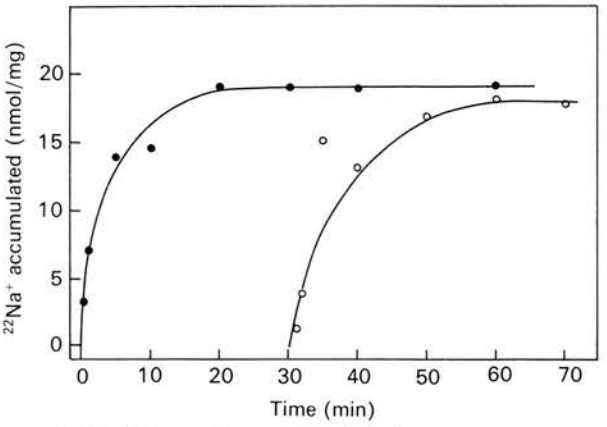


**Fig. 9. $Na^+/Na^+$ exchange by 'ghosts'**

'Ghosts' containing 4 mM-$Na^+$ were incubated at 37 °C in a medium with a similar concentration of $Na^+$ (5 mM) at a protein concentration of 0.12 mg/ml. The medium was supplemented with $^{22}NaCl$ (1.5 $\mu$Ci/ml) either at the start of the incubation (●) or after 30 min (○), and $^{22}Na^+$ accumulation was monitored by filtration of 100 $\mu$l samples.

vary depending on the cation present in the medium, so conditions for calibration curves mimicked experiments as closely as possible.

The quenched fluorescence is restored if $Na^+$ is present in the medium (Fig. 6), as $Na^+$ ions enter the 'ghost' in exchange for $H^+$. As shown in Fig. 6(*a*), there is some leakage of protons if $Na^+$ is replaced by choline. This technique can be used, however, to measure the dependence of proton efflux rate on concentration of $Na^+$, and thus to derive an apparent $K_m$ for $Na^+$ (Fig. 7): we have found a value of $4.7 \pm 0.6$ mM (mean ± S.E.M. for five independent determinations), when using 'ghosts' made with internal pH 6.0, in a medium at pH 7.0. Fig. 6 shows the lack of both quenching and $Na^+$ effects in the absence of a pH gradient, and (Fig. 6*b*) the effect of the ionophore monensin (catalysing the rapid exchange of $Na^+$ for $H^+$), and the lack of effect of the electrogenic protonophore FCCP.

As also shown in Fig. 6(*a*), and analysed in Fig. 7, this technique can be used for the investigation of inhibitors of the exchanger. Amiloride is found to be a competitive inhibitor with $K_i$ $0.26 \pm 0.02$ mM (mean ± S.E.M. for three amiloride concentrations). We also investigated the amiloride derivative ethylisopropylamiloride; however, this was found to have an uncoupling effect in this system, collapsing ΔpH in a concentration-dependent manner in the absence of $Na^+$. This was manifested as a decrease in ACMA quenching when it was present at 10–100 $\mu$M, and an increased rate of proton efflux in the presence of choline chloride (under conditions of Fig. 6). At low concentrations (up to 50 $\mu$M), however, it appeared to have no inhibitory effect on $Na^+$ entry. In assays of 5HT transport, 30 $\mu$M-ethylisopropylamiloride was found to decrease the initial rate of uptake by 70%, consistent with its facilitating proton leakage.

Although not shown, the ACMA-quenching technique can also be used to investigate alternative substrates, by replacing $Na^+$ with other cations. $K^+$ cannot replace $Na^+$, but $Li^+$ is a weak alternative substrate. Initial rates with $Li^+$ are low, however. The $K_m$ for $Li^+$ was approx. 6 mM with a maximum initial rate about one-fifth that found with $Na^+$.

A similar approach can be used to measure $H^+$ entry in response to $Na^+$ efflux (Fig. 8). 'Ghosts' containing 50 mM-$Na^+$ were diluted into $Na^+$-free medium containing ACMA, and the resulting fluorescence quenching was monitored. The experiment shown in Fig. 8 was performed under conditions very similar to those of the experiment recorded in Fig. 5. In Fig. 8 the 30-fold gradient of $Na^+$ sustains a pH gradient of approx. 1.5 units, as measured by the percentage quenching of the ACMA.

Equilibrium is reached after about 10–20 min, and is then sustained for at least a further 30 min under these conditions. At equilibrium, addition of either monensin or FCCP has no effect on the trace, although the quenching is eliminated by adding either $H^+$ or $Na^+$ to the external medium.

Proton entry was found to be sensitive to amiloride (Fig. 8). Obviously one cannot tell from this experiment whether the drug is penetrating into the lumen of the 'ghosts' or is acting on their outer surface.

### $Na^+/Na^+$ exchange

'Ghosts' that contain $Na^+$ exchange this for $^{22}Na^+$ in the medium: this is probably a function of the $Na^+/H^+$ antiporter, already shown to be reversible. This is demonstrated in Fig. 9. 'Ghosts' containing approx. 4 mM-$Na^+$ were incubated in medium containing 5 mM-$^{22}Na^+$, which is accumulated over a period of about 30 min. A similar incubation was performed in the presence of the same concentration of non-radioactive $Na^+$. At 30 min, when, according to the first result, $^{22}Na^+$ uptake had essentially ceased, a trace amount of $^{22}Na^+$ was added to the second incubation. Uptake of radioactivity occurred as in the first incubation, showing that at 30 min there is an equilibrium, with the rate of loss of $Na^+$ from the 'ghosts' equalling the rate of uptake, the $^{22}Na^+$ having equilibrated across the membrane. The plateau value of $^{22}Na^+$ reached in Fig. 9

(18–19 nmol/mg) is equivalent to an internal concentration of 5 mM, if the internal volume of the 'ghosts' is 3.6 $\mu$l/mg (Phillips & Allison, 1978).

Amiloride inhibited $Na^+/Na^+$ exchange (50% decrease in initial rate by 0.5 mM-amiloride; results not shown). This strongly suggests that the exchange is indeed catalysed by the $Na^+/H^+$ antiporter.

## DISCUSSION

We have assayed a novel $Na^+/H^+$ exchange activity of chromaffin-granule membranes by employing a variety of techniques: direct assay of $^{22}Na^+$ accumulation in response to a pH gradient generated by pH jump or by ATP hydrolysis; generation of a pH gradient in response to an imposed $Na^+$ gradient; loss of a pH gradient resulting from $Na^+$ uptake; and $Na^+/Na^+$ exchange. The antiporter has a relatively high $K_m$ for cytosolic (extragranular) $Na^+$. A $K_m$ value of 4.7 mM at pH 7.0 was determined from fluorescence experiments (an indirect measurement of $Na^+$ transport), with values closer to 20 mM being derived from assays using $^{22}Na^+$. $V_{max.}$ values determined from $^{22}Na^+$ transport rates in pH-jump experiments in the absence of ATP were of the order of 150–200 nmol/min per mg; such rates are sustained for only a few seconds, presumably because of rapid loss of the trans-membrane pH gradient. For comparison, $V_{max.}$ of the $H^+$-ATPase of the granule is about 100–200 nmol/min per mg of membrane protein (Johnson *et al.*, 1982; Percy *et al.*, 1985), and that of the catecholamine translocator is about 15 nmol/min per mg (Carty *et al.*, 1985).

Interaction between the $Na^+$ and $H^+$ gradients of the 'ghosts' is demonstrated by the inhibitory effect of $Na^+$ on the initial rate of 5HT transport (Fig. 1). Presumably, when the proton gradient is established from zero as a result of ATP hydrolysis, the presence of $Na^+$ outside the 'ghosts' facilitates proton leakage and hence a decrease in the driving force of 5HT uptake.

The stoichiometry of the exchanger appears to be 1:1. Exchange is unaffected by ionophores that generate potential gradients (e.g. FCCP), so is probably non-electrogenic, and the approximate measurements of equilibrium $H^+$ and $Na^+$ gradients in Figs. 5 and 8 suggest fulfilment of the condition

$$\frac{[H^+]_i}{[H^+]_o} = \frac{[Na^+]_i}{[Na^+]_o} \qquad (2)$$

for the two ion gradients. In these experiments an initial $Na^+$ gradient of about 30-fold supported formation of $H^+$ gradients of about the same magnitude, as measured by both methylamine distribution and ACMA quenching. The size of the $Na^+$ gradient at equilibrium has not been determined in these experiments, but rough measurements suggest that it is not much displaced from the initial value. In contrast with $H^+$ entry, $Na^+$ uptake by the 'ghosts' in response to a pH jump (Fig. 4) produced plateau values of $Na^+$ that were well below those predicted by eqn. (2). This may result from rapid loss of the pH gradient, but even the control values in Fig. 4(*a*) are less than would be expected. This clearly merits a more detailed investigation.

It is not, of course, known whether this equilibrium condition (Eqn. 2) is met *in vivo*, but it seems likely that it is, in view of the relatively high capacity of the exchanger. If the pH gradient across chromaffin-granule membranes in intact cells is about 1.5 pH units (acid inside), a 30-fold gradient of $Na^+$ activity from granule matrix to cytosol could be sustained. The cytosolic concentration of $Na^+$ is unknown, but is presumably of the order of 1–5 mM; the activity coefficient of $Na^+$ in the matrix is also unknown, though is likely to be less than unity (Koppell & Westhead, 1982). The intra-granular concentration of $Na^+$ has been measured by Krieger-Brauer & Gratzl (1982) to be about 47 mM, a value that, though subject to considerable error, would be consistent with the idea that the gradients are in equilibrium.

Ornberg *et al.* (1988) have reported an electron-probe microanalysis of cultured bovine adrenal cells; they suggest that the high granule content of $Na^+$ reported by Krieger-Brauer & Gratzl (1982) is an artefact arising from exchange of intragranular $K^+$ for $Na^+$ *post mortem* and during granule isolation, and that the granule matrix is free of $Na^+$ *in situ*. This seems unlikely from our results, but clearly needs to be investigated further, in view of the low concentrations of $Na^+$ found in 'ghosts' at equilibrium in Fig. 4.

It therefore seems that these secretory vesicles have two transmembrane ion gradients that can be used as energy sources: the $H^+$ gradient that is utilized directly in catecholamine transport, and a $Na^+$ gradient that is coupled to $Ca^{2+}$ transport (Phillips, 1981; Krieger-Brauer & Gratzl, 1982). The granule's membrane potential facilitates electron transport (Njus *et al.*, 1987*b*) and, probably, nucleotide transport (Weber & Winkler, 1981).

Is the $Na^+/H^+$ antiporter identical with the plasma-membrane $Na^+/H^+$ exchanger (reviewed by Aronson, 1985) that has been identified in many cell types? This activity plays a key role in regulation of cytosolic pH, and, through this, of other physiological functions. The specificity and $K_m$ values of the carriers appear similar, as is the $K_i$ value for amiloride (Lazdunski *et al.*, 1985; Seiler *et al.*, 1985). The main difference appears to be the lack of inhibition of the granule exchanger by ethylisopropylamiloride, a highly potent inhibitor of the plasma-membrane activity, showing, for example, a $K_i$ of 22 $\mu$M for exchange by dog cardiac sarcolemmal vesicles (Seiler *et al.*, 1985). This clearly needs further investigation.

This work was supported by project grants from the Medical Research Council and the Wellcome Trust, and by a S.E.R.C. studentship to J. R. H. We gratefully acknowledge the frequent assistance of Dr. D. K. Apps and other members of the laboratory.

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Received 13 May 1988/11 August 1988; accepted 14 September 1988