

**Studies on the mechanism of sodium transport  
at the tonoplast of Beta vulgaris**

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**A thesis submitted to the University of London for  
examination in the degree of Ph.D**

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**To Mum, Dad and Howard**

## Abstract

The aim of the work described in this thesis was to characterise the mechanism of  $\text{Na}^+$  transport at the vacuole membrane (tonoplast) of red beet using isolated tonoplast vesicles. It was confirmed that pure tonoplast vesicles could be prepared from red beet storage root. However, it was found that the KI treatment used in published tonoplast vesicle preparation protocols was deleterious to tonoplast, so this was subsequently modified. Attempts to characterise a  $\text{Na}^+/\text{H}^+$  antiport reported by Blumwald and Poole (1985a; *Plant Physiol.* 78, 163-167) by studying the effect of  $\text{Na}^+$  on pH gradients imposed by a pH jump were not successful. Under the conditions used by these workers the fluorescent pH gradient probe acridine orange misreported the pH gradient and apparently good evidence for a  $\text{Na}^+/\text{H}^+$  antiport was shown to be artefactual. With modifications to the media used for these experiments, acridine orange could be used to monitor the pH gradient and these results were confirmed using another pH gradient probe, [ $^{14}\text{C}$ ]methylamine. However, no evidence was found for a  $\text{Na}^+/\text{H}^+$  antiport as it was not possible to distinguish between changes in the pH gradient due to an  $\text{Na}^+/\text{H}^+$  antiport and changes caused by electrically-coupled  $\text{Na}^+$  and  $\text{H}^+$  fluxes. In an attempt to avoid these problems,  $^{22}\text{Na}$  uptake by tonoplast vesicles was studied in response to pH gradients generated by the  $\text{H}^+$ -ATPase. No pH gradient-dependent  $\text{Na}^+$  uptake was found using this approach. It is concluded from these studies that previous evidence for a tonoplast  $\text{Na}^+/\text{H}^+$  antiport in red beet is artefactual. It is suggested that  $\text{Na}^+$  is passively distributed across the tonoplast in red beet.

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## Acknowledgements

I would like to express my thanks to my supervisors: to Dr. Roger Leigh for guidance throughout the project, and to Prof. George Stewart for his help during the writing-up. A number of people have helped me through discussions and through their senses of humour, particularly Dr. Richard Milling and Dr. Andy Pope. Thankyou to Dave Walker for all the jokes and to Jacqui Marshall and Gethyn Allen for explaining them! I am very grateful to Dr. Lindsey Atkinson for proof reading.

This work was supported by a studentship from the Lawes Agricultural Trust at the Rothamsted Experimental Station.



## Abbreviations

ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
BTP	1,3-bis[tris(hydroxymethyl)methylamino]propane
CAM	crassulacean acid metabolism
Ci	Curie
$\Delta\text{pH}$	transmembrane pH gradient
$\Delta\psi$	membrane potential
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N,N,N',N', tetraacetic acid
ER	endoplasmic reticulum
h	hour
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IDA	iminodiacetate
MES	2-[N-morpholino]ethanesulphonic acid
min	minute
mM	millimolar
nM	nanomolar
NMR	nuclear magnetic resonance
Pi	inorganic phosphate
PPi	inorganic pyrophosphate
PPase	H <sup>+</sup> -translocating inorganic pyrophosphatase
PVPP	polyvinylpolypyrrolidone
RUBISCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecylsulphate
Tris	tris(hydroxymethyl)aminomethane

## **CHAPTER 1: INTRODUCTION**

The work described in this thesis was carried out with the aim of using in vitro techniques to characterise Na<sup>+</sup> transport at the vacuole membrane (tonoplast), particularly in relation to the compartmentation of Na<sup>+</sup> between the cytoplasm and the vacuole. The objectives of this work will be considered in more detail after an introduction to the role of vacuoles.

### **1.1 The Role of Vacuoles in Higher Plants**

The vacuole is the largest organelle in most plant cells, often occupying 90% or more of the protoplast at cell maturity. The vacuole is surrounded by a single membrane, the tonoplast, and is filled with an aqueous sap which has an acidic pH and contains a variety of solutes and some hydrolase enzymes. Vacuoles have a variety of functions and these have been reviewed extensively (e.g. Matile 1978; Marty et al. 1980; Boller and Wiemken 1986; Matile 1987; Raven 1987).

**1.1.1 The vacuole as a way of increasing cell size.** By having a large vacuole filled with water, salts and other solutes, a plant cell is able to increase greatly its size and surface area at relatively low metabolic cost (Wiebe 1978; Raven 1987). The vacuole allows the cell to maintain turgor by accumulating a variety of solutes which do not have to be compatible with cytoplasmic function. A non-vacuolate cell, on the other hand, would require a much higher investment of N in protein and would be restricted to accumulating solutes compatible with cytoplasmic function to maintain turgor. The strategy of vacuolation means that the plant

can greatly increase its size for a given volume of cytoplasm. This allows a larger leaf area to increase the interception of sunlight and uptake of CO<sub>2</sub> for photosynthesis and longer roots to increase inorganic nutrient and water uptake.

**1.1.2 The vacuole as a hydrolytic compartment.** The vacuole has been considered to be a lytic compartment containing hydrolytic enzymes, similar to the lysosomal system of animal cells (Matile 1975; Matile 1978; Boller and Wiemken 1986; Matile 1987).

Cytochemical studies indicated the presence of hydrolytic enzymes in the vacuole and ultrastructural work suggested that the vacuole was able to degrade cytoplasmic components (Matile 1975). However, there is some controversy over this proposed role of the vacuole (Butcher et al. 1977; Leigh 1979; Boller and Wiemken 1986). Butcher et al. (1977) assayed for various hydrolytic enzymes in vacuole lysate and soluble cytoplasmic fractions from Hippeastrum petal protoplasts. They found that esterase, protease, carboxypeptidase,  $\beta$ -galactosidase,  $\alpha$ -glycosidase and  $\beta$ -glycosidase were found in the cytoplasmic fraction rather than the vacuolar fraction. Acid phosphatase, RNase and DNase were present in both fractions. This led them to question the concept of the vacuole as a generalised lysosome.

Most workers, however, have found that hydrolytic enzymes were located in vacuolar rather than cytoplasmic fractions. Nishimura and Beevers (1978) found that protease, carboxypeptidase, phosphodiesterase, RNase, phytase and  $\beta$ -glucosidase were located in isolated vacuoles from castor bean endosperm. They concluded that the vacuole appeared to be the site of hydrolysis of the storage proteins in the seedling. Boller and Kende (1979) examined the

intracellular location of a variety of hydrolytic enzymes in tobacco, tulip and pineapple and found that their location was in the vacuole. They concluded that their results supported the proposed lysosomal role of the vacuole. Boller and Kende (1979) suggested that the discrepancy between their results and those of Butcher et al. (1977) was due to the presence of contaminating hydrolases from the enzymes used to prepare protoplasts in this work. However, Butcher et al. (1977) reported that control experiments were performed to exclude this possibility, so the discrepancy may be a genuine tissue difference.

Direct measurements of protein degradation in vacuoles have been made in addition to studies on enzyme localisation. Nishimura and Beevers (1979) showed that vacuoles isolated from castor bean endosperm protoplasts had been responsible for protein hydrolysis before the vacuoles were isolated and that they continued to hydrolyse protein after isolation. Canut et al. (1985) showed that vacuoles isolated from sycamore protoplasts had acquired protein synthesised before isolation and were able to degrade this protein after isolation.

A lysosomal role for the vacuole has been proposed in chloroplast breakdown in senescing leaves (Wittenbach et al. 1982). Chloroplasts appeared to be taken up and degraded by the vacuole. However, the chloroplast counting methodology used by Wittenbach et al. (1982) is probably unreliable as chloroplasts become increasingly fragile during senescence (Wardley et al. 1984). Wardley et al. (1984) support the view that degradation of chlorophyll and RUBISCO occurs within the chloroplast, which contains a broad spectrum of hydrolytic enzymes.

Vacuoles are formed by endoplasmic reticulum-derived provacuoles fusing and forming an autophagic vacuole around a portion of cytoplasm, which is digested by hydrolytic enzymes. These autophagic vacuoles fuse to form the central vacuole of the mature cell (Marty 1978; Marty et al. 1980). The autophagic role of developing vacuoles is clearly important in germinating seeds, where the developing vacuoles are responsible for the hydrolysis of storage proteins (Nishimura and Beevers 1978; 1979). The role of the mature vacuole in intracellular digestion may actually be quite limited (Marty et al. 1980). Although mature vacuoles hydrolyse protein (Canut et al. 1985), it is not yet clear how this fits into the overall picture of cell protein turnover. In mature cells the function of the vacuole in solute compartmentation may well be more important than any lytic function (Marty et al. 1980).

**1.1.3 The vacuole as a long-term storage compartment.** Vacuoles contain compounds that appear to remain in the vacuole throughout the life of the cell. Pigments, such as betanin in red beet (Leigh et al. 1979) or anthocyanins in petals (Wagner 1979), are located in the vacuole. Some plants accumulate compounds in the vacuole, such as glucosinolates (Helminger et al. 1983) or glycosides (Saunders and Conn 1978), that would be toxic in the cytoplasm as enzymes there would convert them to harmful products. These compounds function as a means of defence against attack by herbivores and insects (Matile 1987). On disruption of the tissue by herbivore or insect attack, cytoplasmic enzymes hydrolyse these compounds into poisonous products.

1.1.4 The vacuole as a homeostat and reversible store of nutrients. Cytoplasmic pH is strictly controlled in plants (Smith and Raven 1979; Davies 1986; Kurdjian and Guern 1989) and the vacuole plays an important role in pH homeostasis by acting as a buffer against cytoplasmic pH change. Using  $^{31}\text{P}$  nuclear magnetic resonance (NMR) it is possible to estimate cytoplasmic and vacuolar pH in vivo to within 0.1 pH units (Kime et al. 1981; Roberts 1984). The resonance peak of phosphate is shifted by pH so the vacuolar and cytoplasmic pools of phosphate can be resolved and changes in pH in the two compartments followed. Torimitsu et al. (1984) incubated root tips in buffers of different pH and used  $^{31}\text{P}$  NMR to monitor vacuolar and cytoplasmic pH. When the external pH was varied between 3 and 10, the vacuolar pH varied between 4.4 and 7.4 but the cytoplasmic pH was kept between 6.8 and 7.4. Although the importance of the vacuole in cytoplasmic pH regulation is indicated by such experiments, little is understood about the regulation of  $\text{H}^+$  fluxes between cytoplasm and vacuole. This will presumably involve regulation of the ATP- and  $\text{PPi}$ -dependent  $\text{H}^+$ -pumps at the tonoplast. However, as the vacuole has a finite capacity for  $\text{H}^+$  regulation, it can only serve to buffer changes in cytoplasmic pH in the short term. In the long term the cell must adjust  $\text{H}^+$  fluxes across the plasma membrane.

Vacuoles are involved in the intracellular distribution of photo-assimilates. In the storage roots of red beet sucrose is stored in the vacuole (Doll et al. 1979; Leigh et al. 1979). The invertase that is responsible for the first step in the mobilisation of sucrose is also found in the vacuole when sucrose is mobilised (Leigh et al. 1979). In sugar cane, sucrose storage is predominantly vacuolar (Thom et al. 1982).

In barley mesophyll cells the vacuole serves as a short-term store for photosynthate. Much of the fixed carbon is metabolised to sucrose in the cytosol and is then transported to the vacuole (Kaiser et al. 1982). A sucrose transporter in the tonoplast (Kaiser and Heber 1984) is thought to allow the maintenance of equal concentrations of sucrose in the two compartments so that the cytoplasm and vacuole are balanced osmotically. The vacuoles of barley mesophyll appear to function as a temporary reserve of sucrose while the rate of photosynthesis exceeds the rate of export to the phloem. However, not all plants use the vacuole as a temporary store of photosynthate: in Melilotus mesophyll protoplasts sucrose is excluded from the vacuole (Boller and Alibert 1983).

The vacuole plays a very important role in plants with crassulacean acid metabolism (CAM). Plants with CAM fix CO<sub>2</sub> during the night and store it as malic acid in the vacuole. During the day the stored malic acid is returned to the cytoplasm where it is decarboxylated to provide CO<sub>2</sub> for photosynthesis. This strategy reduces water loss as gas exchange takes place during the night rather than the day (see Ting 1984 and Lüttge 1987 for recent reviews). The large quantities of malate synthesised at night in the cytosol are transported to the vacuole so that essentially all the malic acid is present in the vacuole (Buser and Matile 1977). This compartmentation is maintained throughout the diurnal cycle (Kenyon et al. 1978). A malate carrier has been demonstrated in isolated intact vacuoles (Buser-Suter et al. 1982). Experiments with isolated tonoplast vesicles suggest that malate is accumulated in the vacuole in response to an inside-positive membrane potential generated by ATP- and P<sub>Pi</sub>-dependent H<sup>+</sup>-pumps at the tonoplast (Jochem and Lüttge 1987; Marquardt and Lüttge 1987; Struve and Lüttge 1987).

Malate is transported through a carrier in response to this membrane potential with a high specificity (White and Smith 1989). The action of these transport systems leads to malic acid accumulation in the vacuole. Efflux of malic acid is a passive process (Lüttge et al. 1975). However, how the transport of malic acid is regulated is still unclear.

The cytoplasmic phosphate concentration is closely controlled by the vacuole. Experiments with  $^{31}\text{P}$  NMR have given useful information on the compartmentation of phosphate between cytoplasm and vacuole in vivo. The phosphate concentrations in the cytoplasm and vacuole are estimated by measuring the area under the resonance peaks corresponding to cytoplasm and vacuole. In root tissue (Lee and Ratcliffe 1983), leaf tissue (Waterton et al. 1983; Foyer and Spencer 1986) and cultured cells (Rebeille et al. 1983) the vacuolar phosphate concentration varies according to the phosphate supply whereas the cytoplasmic concentration is kept constant. The vacuole appears to act as a buffer to maintain a constant cytoplasmic concentration. Although the response of phosphate compartmentation to phosphate status is well documented, little is currently understood about the mechanisms of phosphate transport at the tonoplast or their regulation.

The vacuole is known to be a major nitrate storage pool (Martinoia et al. 1981; Granstedt and Huffaker 1982). It is tempting to speculate that the vacuole is able to control the cytoplasmic nitrate concentration in the same way as for phosphate. However, as yet there are no direct measurements of the way cytoplasmic and vacuolar nitrate concentrations respond to different levels of nitrate nutrition.



There is a strict compartmentation of  $\text{Ca}^{2+}$  between vacuole and cytoplasm in plant cells. Studies with the  $\text{Ca}^{2+}$ -sensitive dye aequorin in giant algal cells (Williamson and Ashley 1982) and with efflux analysis (Macklon 1984) suggest that cytosolic  $\text{Ca}^{2+}$  levels are in the range 0.1 to 1  $\mu\text{M}$  whereas vacuolar concentrations can be up to 10 mM. More recently, work with  $\text{Ca}^{2+}$ -sensitive microelectrodes in giant algal cells has confirmed that cytosolic  $\text{Ca}^{2+}$  levels are very low (Miller and Sanders 1987). Although there have been few measurements made on cytosolic  $\text{Ca}^{2+}$  in higher plant cells, there is much interest as to its possible role as a regulator (Hepler and Wayne 1985). The role of  $\text{Ca}^{2+}$  in the vacuole appears to be in contributing to the osmotic potential of the cell sap and hence turgor generation (Leigh et al. 1986).

It is considered that the cytoplasmic concentrations of  $\text{K}^+$  and  $\text{Na}^+$  are also regulated by the vacuole. This will be discussed in more detail below as it forms the physiological background to the work carried out in this thesis.

## **1.2 The Role of $\text{Na}^+$ in Plant Cells in Relation to Cation Supply and its Compartmentation between Cytoplasm and Vacuole**

Sodium is not required as a macronutrient by most plants and its role as a micronutrient appears to be restricted to some  $\text{C}_4$  plants (Wyn Jones and Pollard 1983). Nevertheless, the role of  $\text{Na}^+$  in plants is of considerable interest, particularly in the response of plants to K-deficient soils and to high levels of salinity.

**1.2.1 The compartmentation of Na<sup>+</sup> between cytoplasm and vacuole in relation to K<sup>+</sup> supply.** Although K<sup>+</sup> is an essential nutrient and is distributed throughout the plant cell, it has different functions in the cytoplasm and vacuole (Leigh and Wyn Jones 1984, 1986; Flowers and Läuchli 1983). Vacuolar K<sup>+</sup> is present as salts such as KCl, KNO<sub>3</sub> and K malate that make a large contribution to the osmotic potential of the cell sap and hence turgor generation but K<sup>+</sup> in the vacuole does not appear to have any biochemical functions. In the cytoplasm K<sup>+</sup> is required to activate many enzymes, including those involved in glycolysis, protein synthesis and photosynthesis (Wyn Jones et al. 1979).

The cytoplasmic and vacuolar pools of K<sup>+</sup> are thought to behave very differently in their response to K<sup>+</sup> deficiency. The vacuole acts as a buffer to maintain the high cytoplasmic K<sup>+</sup> concentration required for metabolism. As the tissue level of K<sup>+</sup> declines when there is an inadequate K<sup>+</sup> supply the concentration in the vacuole declines in order to maintain the cytoplasmic concentration. The vacuole accumulates Na<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>, according to their availability, to maintain its osmotic potential. If these alternative cations are not available organic solutes must be accumulated instead. There is a large body of indirect evidence in favour of this model (Leigh and Wyn Jones 1984, 1986). More direct evidence, based on estimations of cytoplasmic and vacuolar cation levels, largely comes from studies on barley.

Jeschke and Stelter (1976) estimated cytoplasmic and vacuolar ion concentrations in barley roots by comparing the ion contents of non-vacuolated meristems with highly-vacuolated regions further from the root tip. It is assumed that the ion content of the meristem

reflects that of the cytoplasm whereas the ion content of highly vacuolated cells reflects that of the vacuole. When roots were grown without  $K^+$ , the cytoplasmic  $K^+$  concentration was 110 mM, whereas the vacuolar concentration was only 20 mM. When 1 mM  $Na^+$  was supplied to the roots in the absence of  $K^+$ ,  $Na^+$  was accumulated to 100 mM in the vacuole, whereas the cytoplasmic concentration was only 15 mM. Huang and van Steveninck (1989) used a similar profile analysis to deduce cytoplasmic and vacuolar  $K^+$  concentrations, except that ion concentrations were measured using X-ray microanalysis rather than atomic absorption. In high- and low-salt barley roots the cytoplasmic  $K^+$  concentration was around 200 mM. The vacuolar concentration was around 200 mM in high-salt roots but only 20 mM in low-salt roots. Compartmentation of cations in response to  $K^+$  supply has also been measured within individual barley leaf cells using X-ray microanalysis (Leigh et al. 1986). When plants were grown in full nutrient solution  $K^+$  was localised in both cytoplasm and vacuole of leaf cells, whereas when  $K^+$  supply was restricted no  $K^+$  was detectable in the vacuole. Under these conditions mesophyll vacuoles accumulated  $Na^+$  and  $Mg^{2+}$ , whereas epidermal vacuoles accumulated  $Ca^{2+}$ . The growth of K-deficient barley was reduced less when  $Na^+$  was available and  $Na^+$  was then accumulated in preference to  $Mg^{2+}$  or  $Ca^{2+}$ . The accumulation of  $Na^+$  by the vacuole in K-deficient conditions thus minimises reduction in growth as a result of K-deficiency.

**1.2.2 The compartmentation of  $Na^+$  between cytoplasm and vacuole in response to a saline environment.** The responses of plants to saline conditions are of much interest as many crop plants are sensitive to high concentrations of salt in the soil. In parts of the world where crops need to be irrigated, salinization of the soil

has become a real problem (Flowers et al. 1977). The way in which different plants respond to saline conditions varies considerably, but compartmentation of  $\text{Na}^+$  between cytoplasm and vacuole is of interest in a wide range of plant species (Wyn Jones et al. 1977; Flowers et al. 1977; Greenway and Munns 1980; Yeo 1983; Flowers et al. 1986; Cheeseman 1988). Plants have been divided into two main categories on the basis of how they respond to saline soils; halophytes and glycophytes. Halophytes are tolerant of salt in the environment and usually their growth is stimulated by  $\text{NaCl}$ . The optimum  $\text{NaCl}$  concentration can be several hundred millimolar for some species. Glycophytes, on the other hand, are relatively sensitive to salt, and the growth of especially salt-sensitive species can be greatly reduced by as little as 50 mM  $\text{NaCl}$ . In fact, there is no sharp dividing line between halophytes and glycophytes and a whole spectrum of plant responses to salt is found.

The presence of high concentrations of salt in the soil presents the plant with a water relations problem as the water potential of the soil water is lowered. The plant must be able to lower its tissue water potential by accumulating solutes to avoid a water deficit. However, it must avoid accumulating ions to toxic concentrations in the cytoplasm. Somehow, the plant must strike a balance between avoiding a water deficit and avoiding excess ion toxicity.

Halophytes respond to the saline environment that they are adapted to grow in by accumulating ions to keep a sufficiently low tissue water potential. A well-studied example is Suaeda maritima. It accumulates  $\text{Na}^+$  and  $\text{Cl}^-$  to concentrations of over 400 mM. Studies with electron probe X-ray microanalysis on root and leaf

tissue have shown that it avoids ion toxicity by compartmentation of  $\text{Na}^+$  and  $\text{Cl}^-$  between cytoplasm and vacuole (Harvey et al. 1981; Hajibagheri and Flowers 1989). When grown in the presence of 200 mM NaCl, vacuolar concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  were around 400 mM in root cortical cells, whereas cytoplasmic concentration of these ions were only about 100 mM (Hajibagheri and Flowers 1989). The osmotic potential of the cytoplasm is balanced by accumulation of solutes compatible with metabolism, such as glycinebetaine (Hall et al. 1978).

Plants that are less well adapted to a saline environment tend to respond to salt stress by excluding ions from the shoots to avoid ion toxicity and accumulating organic solutes in the shoot to avoid a water deficit (Greenway and Munns 1980). Reabsorption of salt from the xylem sap by xylem parenchyma cells in the root may be important in controlling accumulation of salt by the shoot (Yeo et al. 1977). Ion transport to vacuoles in the root can limit net transport to the shoot by reducing the cytoplasmic ion concentration (Hajibagheri et al. 1987). When plants with this strategy, such as maize, are unable to exclude salt from the shoot, growth is usually reduced. Hajibagheri et al. (1987) compared a salt-sensitive with salt-tolerant varieties of maize and showed that the salt-tolerant varieties had lower shoot concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  than the salt-sensitive variety. Measurements with X-ray microanalysis showed there was also a more efficient compartmentation of  $\text{Na}^+$  into the vacuole in the roots of the salt-tolerant varieties, giving a lower cytoplasmic  $\text{Na}^+$  concentration. This would account for the reduced  $\text{Na}^+$  transport to the shoot in the salt-tolerant varieties.

Barley is another example of a plant that appears to use

compartmentation of Na<sup>+</sup> between cytoplasm and vacuole as part of its strategy for dealing with Na<sup>+</sup> in the soil. Barley roots are able to maintain a high K/Na ratio in the cytoplasm when the concentration of Na<sup>+</sup> available to the roots exceeds that of K<sup>+</sup> (Pitman et al. 1981; Jeschke and Stelzer 1976). Under such conditions Na<sup>+</sup> is accumulated in the vacuole. This, together with extrusion of Na<sup>+</sup> from the cytoplasm by the plasma membrane, maintains a low cytoplasmic Na<sup>+</sup> concentration and hence reduces transport of Na<sup>+</sup> to the shoot.

1.2.3 Na<sup>+</sup> transport at the tonoplast. The above examples of the compartmentation of Na<sup>+</sup> between cytoplasm and vacuole illustrate the importance of the Na<sup>+</sup> transport properties of the tonoplast. The tonoplast must provide a barrier to Na<sup>+</sup> movement to allow the cytoplasm and vacuole to behave as distinct compartments yet also energise Na<sup>+</sup> transport into the vacuole against its concentration gradient. Although little is known about the mechanism of Na<sup>+</sup> transport at the tonoplast, the aim of the work described in this thesis was to increase our understanding of this by using isolated tonoplast vesicles. Studies of this kind have greatly increased our understanding of other transport systems at the tonoplast (1.3).

### 1.3 The Study of Solute Transport at the Tonoplast

Although the use of in vivo techniques such as steady-state tracer efflux analysis (Pitman 1963; Pierce and Higinbotham 1970; Mills et al. 1985) and <sup>31</sup>P NMR (1.1.4) has given some information on the fluxes of solutes between cytoplasm and vacuole, more detailed studies of transport mechanisms have been made with in

vitro techniques.

**1.3.1 In vitro transport studies with isolated intact vacuoles or tonoplast vesicles.** In the mid 70's it became possible to work on preparations of intact vacuoles from mature plant tissue after Wagner and Siegelman (1975) and Leigh and Branton (1976) developed protocols for their isolation. Intact vacuoles offer advantages over in vivo studies as transport in and out of the vacuole can be studied without other intracellular compartments or the plasma membrane complicating interpretation of data. Also the medium on the cytoplasmic face of the tonoplast can be controlled by the experimenter and permit more detailed characterisation of transport processes.

These studies allowed marker enzymes for the tonoplast to be characterised, which has in turn allowed methods to be developed for isolating tonoplast vesicles from tissue homogenates (Churchill et al. 1983; Bennett et al. 1984; Poole et al. 1984). Vesicles have several advantages over intact vacuoles for transport studies: 1) they are essentially devoid of endogenous solutes; 2) they can be loaded with media of the desired composition; 3) as vesicles are much smaller than vacuoles (0.1  $\mu\text{m}$  across) they have a much larger surface area/volume ratio so observed rates of transport are faster, making experimental investigation easier; 4) yield of membranes can be higher than with intact vacuole preparations.

**1.3.2 Primary transport at the tonoplast.** Over the last few years evidence has emerged that solute transport across the tonoplast is driven by an electrochemical proton gradient (Poole 1978; Sze 1985; Marin 1985). Studies with isolated intact vacuoles showed that there

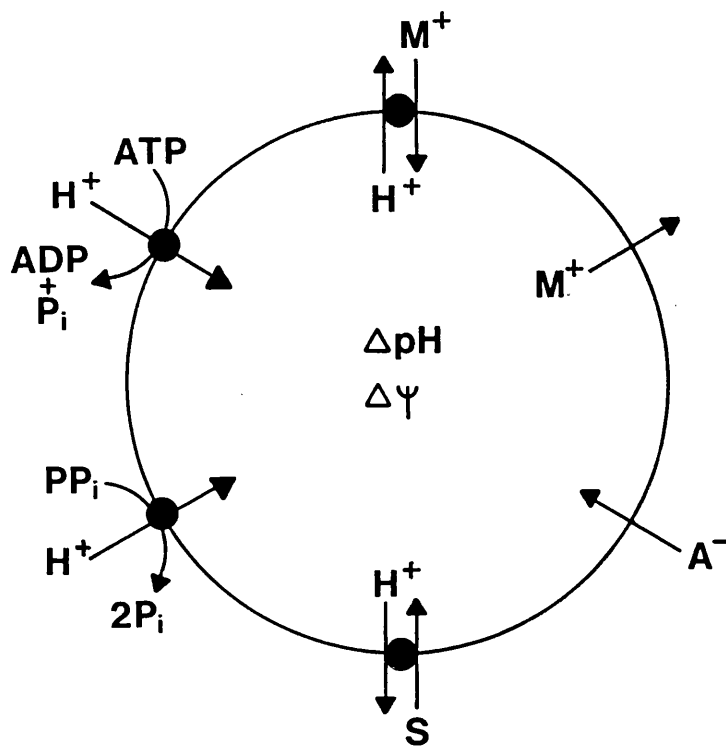
is an ATPase and a PPase at the tonoplast (Walker and Leigh 1981a,b). It has become clear that from studies with vesicles that the vacuolar ATPase and PPase are H<sup>+</sup>-pumps (Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985). The ATP- and PPi-dependent H<sup>+</sup>-pumps are different enzymes that can be separated chromatographically after solubilisation from the membrane (Rea and Poole 1986; Wang et al. 1986). The H<sup>+</sup>-ATPase pumps H<sup>+</sup> from cytoplasm to vacuole, which provides an electrochemical H<sup>+</sup> gradient across the tonoplast to drive solute transport by secondary transport systems (Sze 1985; Rea and Sanders 1987). However, the function of the H<sup>+</sup>-PPase is less clear as it is not known whether the enzyme works in vivo to hydrolyse or synthesise PPi. The H<sup>+</sup>-PPase may act to stabilise the cytosolic PPi concentration (Rea and Sanders 1987). It is also possible that this enzyme could transport K<sup>+</sup> as well as H<sup>+</sup> and so function as a primary K<sup>+</sup> pump.

**1.3.3 Secondary transport at the tonoplast.** The protonmotive force generated at the tonoplast consists of two components, a pH gradient (inside-acid) and a membrane potential (inside-positive). The relationship between these two components (expressed in millivolts at 25°C) is

$$\begin{aligned}\Delta p &= \Delta\psi - 60[\text{pH}_i - \text{pH}_o] \\ &= \Delta\psi - 60 \Delta\text{pH}\end{aligned}$$

where  $\Delta p$  is the protonmotive force in mV,  $\Delta\psi$  the membrane potential measured in mV,  $\text{pH}_i$  the pH inside the vacuole and  $\text{pH}_o$  the pH outside the vacuole so that  $\Delta\text{pH}$  is the pH gradient across the membrane. The pH gradient and the membrane potential provide the driving force for secondary transport systems (Fig. 1.1).





**Fig. 1.1. Primary and secondary transport systems at the tonoplast. The electrogenic proton pumps generate a pH gradient ( $\Delta\text{pH}$ ) and a membrane potential ( $\Delta\psi$ ). Anions ( $\text{A}^-$ ) are accumulated in response to the membrane potential whereas accumulation of cations ( $\text{M}^+$ ) and sucrose ( $\text{S}$ ) is driven by the pH gradient via antiport systems. Export of cations and anions may be in response to changes in ion channel activity and membrane potential.**

Uptake of anions such as chloride and nitrate is driven by the membrane potential (Churchill and Sze 1984; Pope and Leigh 1987; Kaestner and Sze 1987). Uptake of  $\text{Na}^+$  is driven by a  $\text{Na}^+/\text{H}^+$  antiport (Blumwald and Poole 1985a; Garborino and DuPont 1988). Similarly uptake of  $\text{Ca}^{2+}$  is driven by a  $\text{Ca}^{2+}/\text{H}^+$  antiport (Bush and Sze 1986; Blumwald and Poole 1986). Uptake of sucrose is driven by a sucrose/ $\text{H}^+$  antiport (Briskin et al. 1985). Recent electrophysiological studies have revealed voltage- and  $\text{Ca}^{2+}$ -regulated ion channels with a broad specificity for anions and cations (Coyaud et al. 1987; Hedrich and Neher 1987). A nitrate/ $\text{H}^+$  symport reported by Blumwald and Poole (1985b) and thought to function in nitrate export was subsequently shown to be an

experimental artefact by Pope and Leigh (1988a).

**1.3.4 Approaches for measuring transport processes at the tonoplast.** A variety of methods can be used to study transport processes in vesicles. The two components of the protonmotive force can be measured using fluorescent or radioactive probes. In general, fluorescent probes have the advantage over radioactive probes of better time resolution and convenience but are more prone to artefacts.

An inside-acid pH gradient can be followed by measuring the quenching of fluorescent probes such as quinacrine, acridine orange or 9-amino acridine or the accumulation of a radiolabelled weak base such as [ $^{14}\text{C}$ ]methylamine (Deamer et al. 1972; Lee and Forte 1978; Lee et al. 1982; Kashket 1985). These probes are amines which are accumulated inside acidic membrane-bound compartments by ion trapping. The non-protonated form of the probe is freely permeable across the membrane whereas the protonated form has a much lower permeability. This means that as the probe is protonated in the more acidic environment it will be trapped inside and accumulated to a higher concentration. The accumulation of a radioactive probe can be measured after filtration of the vesicles from the surrounding medium. The accumulation of a fluorescent probe is measured as a quench in fluorescence, which is probably caused by the probe molecules stacking together inside the vesicles.

The membrane potential can also be monitored using fluorescent probes such as Oxonol V or by measuring the uptake of a radiolabelled permeant anion, such as thiocyanate (Waggoner 1979; Bashford and Smith 1979; Kashket 1985).

Secondary transport can be measured directly by monitoring the ion of interest through radioisotopes such as  $^{36}\text{Cl}$  or through fluorescent probes sensitive to the solute under study such as the chloride-sensitive fluorescent probe SPQ (Pope and Leigh 1988b). Secondary transport can also be studied indirectly by measuring the pH gradient or membrane potential that drives solute movement. For example, uptake of anions causes a dissipation of the membrane potential generated by the  $\text{H}^+$ -pumps and so causes a stimulation of pH gradient formation as the two components of the proton motive force are equivalent. Stimulation of pH gradient formation and dissipation of the membrane potential by anions have been used to study anion transport in this way (Pope and Leigh 1987; Kaestner and Sze 1987). The approaches that can be used for studying  $\text{Na}^+$  transport are considered in more detail below.

#### 1.3.5. Techniques for studying $\text{Na}^+$ transport at the tonoplast.

Blumwald and Poole (1985a) found evidence for a  $\text{Na}^+/\text{H}^+$  antiport at the tonoplast of red beet by studying the effect of  $\text{Na}^+$  on pH gradients. The pH gradients were imposed artificially by a 'pH jump' technique, where tonoplast vesicles are loaded with buffer at pH 6.0 and diluted into buffer at pH 8.0 to impose a pH gradient. The pH gradient was monitored with the fluorescent probe acridine orange and it was found that addition of  $\text{Na}^+$  led to a collapse of the pH gradient measured by this probe. Rea et al. (1987b) made similar observations using the pH jump technique but also measured uptake of  $^{22}\text{Na}$  directly in response to a pH gradient. Garborino and DuPont (1988) measured a  $\text{Na}^+/\text{H}^+$  antiport in vesicles from barley roots, using the tonoplast  $\text{H}^+$ -ATPase to generate a pH gradient and a similar approach has also been used in intact vacuoles (Blumwald et al. 1987). The pH jump technique has the advantage that observations

on the effect of  $\text{Na}^+$  are not complicated by the ability of the ATPase to compensate for changes in the pH gradient and that a known pH gradient can be imposed. This technique is therefore more suitable for kinetic studies. The approach of imposing gradients artificially can also be used to study pH gradient formation in response to an outwardly-directed  $\text{Na}^+$  concentration gradient (Blumwald and Poole 1985a; Rea et al. 1987b).

#### 1.4 Research Objectives

The work described in this thesis was carried out with the purpose of further characterising the  $\text{Na}^+/\text{H}^+$  antiport at the tonoplast of red beet by using isolated tonoplast vesicles. The aim of this characterisation was to understand in more detail how the compartmentation of  $\text{Na}^+$  between cytoplasm and vacuole is related to the  $\text{Na}^+$  transport properties of the tonoplast. It was intended to use artificially-imposed pH and  $\text{Na}^+$  gradients in these experiments for the reasons discussed above.

There were a number of reasons for choosing tonoplast vesicles from red beet as a system for study. Transport-competent tonoplast vesicles can easily be isolated from red beet tissue homogenates (Bennett et al. 1984; Poole et al. 1984). Although it was intended to use vesicles rather than isolated intact vacuoles for this work, it would also be possible to use intact vacuoles from this tissue (Leigh and Branton 1976) in the event of unforeseen problems specifically associated with vesicles. At the start of this work, the  $\text{Na}^+/\text{H}^+$  antiport at the tonoplast of red beet had been studied in more detail than any other plant (Blumwald and Poole 1985a; Rea et al.

1987b). An interesting feature of the red beet tonoplast  $\text{Na}^+/\text{H}^+$  antiport is its activation by washing of discs of beet in aerated distilled water (Rea et al. 1987b). Under these conditions beet discs develop the ability to accumulate  $\text{Na}^+$  selectively in the presence of  $\text{K}^+$  (Poole 1971), so the results of Rea et al. (1987b) suggest that the tonoplast  $\text{Na}^+/\text{H}^+$  antiport may be responsible for this. This suggested that red beet tonoplast vesicles from washed discs could be used as a model system to see if changes in the  $\text{Na}^+$  transport properties at the tonoplast were responsible for changes in the tissue K/Na ratio.

**2.1 Introduction**

As it was intended to use artificially-imposed pH and Na<sup>+</sup> gradients to study the Na<sup>+</sup>/H<sup>+</sup> antiport (1.4), it was important to use a purified tonoplast vesicle preparation. This is because all sealed vesicles will participate in experiments of this kind. If the tonoplast preparation was contaminated by a membrane with a Na<sup>+</sup> transport system, results from such experiments would be misleading. If it is intended only to study transport in response to the protonmotive force generated by the primary proton pumps, purity is arguably less important. This is because the tonoplast can be selectively energized, either by using the PPase unique to the tonoplast, or by using the ATPase in the presence of inhibitors of the plasma membrane and mitochondrial ATPases.

Marker enzyme analysis of red beet tonoplast vesicles purified from microsomal membranes on sucrose step gradients suggested that relatively pure preparations were obtained by this method. The ATPase activity associated with this fraction (Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985) has very similar characteristics to the ATPase associated with isolated intact vacuoles (Walker and Leigh 1981a). Similarly, the characteristics of the PPase (Rea and Poole 1985) correspond to those observed in isolated intact vacuoles (Walker and Leigh 1981b). The plasma membrane marker, vanadate-sensitive ATPase, and the mitochondrial marker, azide or oligomycin-sensitive ATPase are either absent or present at very low activity (Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985).

The procedures for isolating tonoplast vesicles referred to above involved washing the microsomal membranes with 0.25 M KI before separating the tonoplast vesicles on sucrose step gradients. Potassium iodide is a chaotropic salt which is able to dissociate peripheral proteins from membranes by disrupting hydrophobic interactions (Hatefi and Hanstein 1974). The KI wash was originally developed to reduce contamination of plasma membrane preparations from red beet by non-specific phosphatase (Briskin and Poole 1983) and was subsequently used during the preparation of tonoplast vesicles (Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985). The inclusion of the KI wash in the tonoplast vesicle preparation procedure was intended to reduce contamination by non-specific phosphatase, which could complicate the interpretation of ATPase assays (Poole et al. 1984). It has also been suggested that a wash with 0.25 M KI during the preparation of tonoplast from sugar beet is necessary to reduce contamination by mitochondrial membrane fragments (Rea et al. 1987a).

However, it was possible that the KI wash had less beneficial effects. Bennett et al. (1984) suggested that the mitochondrial  $F_1$ -ATPase (the azide- and oligomycin-sensitive ATPase) was lost from mitochondrial membranes during the KI wash. This would mean that absence of this marker may not necessarily indicate the absence of mitochondrial membranes. As previous marker enzyme studies on red beet tonoplast vesicles involved looking for  $F_1$ -ATPase activity in vesicles isolated from KI-washed microsomal membranes it was felt important to exclude the possibility that tonoplast vesicles prepared in this way were contaminated with mitochondrial membranes. It was also possible that the KI wash was removing peripheral proteins involved with solute transport at the tonoplast. Because of

this, the procedure of isolating tonoplast vesicles on sucrose step gradients was investigated, both for purity and for the effect of the KI wash procedure. The results presented in this chapter show that tonoplast vesicles prepared in this way are purified, but that the KI wash is deleterious to tonoplast.

The purity of the tonoplast vesicle fraction was assessed by comparing the activities of marker enzymes with the activities in the microsomal fraction. This assessment of purity is therefore relative to the microsomal fraction rather than absolute.



## 2.2 Materials and Methods

2.2.1 Plant material and chemicals. Red beet (Beta vulgaris L. cv. Detroit Crimson Globe), were grown in a glasshouse and storage roots harvested immediately before use.

ATP and PPI were converted from their sodium salts to their BTP salts by cation-exchange chromatography with Dowex-50W-X8 resin (H<sup>+</sup>-form) and titration with BTP. Nitrate-BTP was prepared by titration of nitric acid with BTP.

2.2.2 Membrane preparation. The method of Poole et al. (1984), as modified by Rea and Poole (1985), was used as a basis.

The homogenisation medium contained 0.65 M ethanolamine, 0.28 M choline chloride, 10 mM  $\alpha$ -glycerophosphate, 2 mM salicylhydroxamic acid, 0.5 mM butylated hydroxytoluene, 0.2% (w/v) BSA, 3 mM EDTA, 25 mM potassium metabisulphite, 5 mM DTT, 10% (w/v) PVPP and 70 mM Tris-sulphate pH 8.0. Choline chloride and ethanolamine were included to inhibit phospholipase D and glycerophosphate to inhibit phosphatidic acid phosphatase (Scherer and Morre 1978), salicylhydroxamic acid and butylated hydroxytoluene as antioxidants, BSA to bind free fatty acids, EDTA to chelate heavy metals, potassium metabisulphite as a reductant, DTT to keep -SH groups reduced and PVPP to bind polyphenols. The resuspension medium contained 1.1 M glycerol, 1 mM EDTA, 0.5 mM butylated hydroxytoluene, 2 mM DTT, and 5 mM BTP-MES pH 8.0. The 0.3 M KI solution and 10% and 23% (w/w) sucrose solutions were made in resuspension medium. During the membrane preparation, all operations were carried out on ice, or in the case of centrifuge runs, at 4°C.

Chilled, diced red beet storage root (330g) was blended with 330 ml ice-cold homogenisation medium in a Kenwood Chef food processor at a medium speed setting. The brei was squeezed through 2 layers of cheesecloth to remove large tissue fragments, and then the filtrate was filtered through 8 layers of cheesecloth to remove fine debris. This filtrate was centrifuged at 10,000 g ( $r_{av}$ ) for 15 min to pellet cell walls, nuclei and mitochondria, then the supernatant was centrifuged at 85,000 g ( $r_{av}$ , Beckman Type 35 rotor, 33,000 rpm) for 25 min to obtain a microsomal pellet. The microsomes were resuspended in resuspension medium using a hand-held glass-in-glass pestle homogeniser. The microsomal suspension was washed by diluting the resuspended membranes with resuspension medium with or without 0.3 M KI and centrifuging at 64,000 g ( $r_{av}$ , Beckman SW 27 rotor, 22,000 rpm) for 30 min. For the experiments in which a KI wash was used, the final KI concentration was 0.25 M. The washed pellets were resuspended as above and layered onto 10/23% (w/w) sucrose step gradients and centrifuged at 66,000 g ( $r_{av}$ , Beckman SW 27.1 rotor, 22,000 rpm) for 2 h. Tonoplast vesicles were removed from the interface with a Pasteur pipette, diluted with resuspension medium, pelleted at 66,000 g ( $r_{av}$ , Beckman SW 27.1 rotor) for 30 min and, finally, suspended in resuspension medium. For experiments in which the effect of omitting a wash procedure was investigated, an aliquot of the microsomal suspension was kept on ice while the wash procedures were carried out.

Potassium iodide washed- and resuspension medium washed- microsomal membranes were also fractionated on 0/50% (w/w) linear sucrose gradients prepared by the method of Stone (1974). After centrifugation at 66,000 g ( $r_{av}$ , Beckman SW 27.1 rotor) for 2 h, the gradients were divided into 1 ml fractions by piercing the

bottom of the tube and collecting drops. The sucrose contents of the fractions were determined using a refractometer. All fractions were stored at  $-20^{\circ}\text{C}$  before assay.

**2.2.3 Enzyme assays.** ATPase activity was determined by measuring the release of inorganic phosphate after a 1 h incubation at  $25^{\circ}\text{C}$  in 0.5 ml of 250 mM glycerol, 50 mM KCl, 1.5 mM ATP-BTP, 1.5 mM  $\text{MgSO}_4$ ,  $0.25 \mu\text{g ml}^{-1}$  gramicidin D and 20 mM BTP-MES. The assay conditions were chosen to ensure near-maximal activity. Hence, vanadate-sensitive ATPase was assayed at pH 6.5 (Briskin and Poole 1983), whereas azide-sensitive and nitrate-sensitive ATPase were assayed at pH 8.0 (Wang and Sze 1985; Poole et al. 1984). Potassium chloride was included as the tonoplast ATPase is stimulated by  $\text{Cl}^{-}$  (Walker and Leigh 1981a; Poole et al. 1984) and the plasma membrane ATPase is stimulated by  $\text{K}^{+}$  (Briskin and Poole 1983). The ionophore gramicidin was included to prevent formation of pH gradients, which would constrain  $\text{H}^{+}$ -ATPase activity in vesicles sealed to  $\text{H}^{+}$ . Where used, vanadate was added as  $50 \mu\text{M Na}_3\text{VO}_4$ , azide as 1 mM  $\text{NaN}_3$  and nitrate as 50 mM nitrate-BTP (pH 8.0). Reaction was started by the addition of vesicles (1-4  $\mu\text{g}$  protein). Phosphate released was determined by the method of Ames (1966). Addition of the Ames acid-molybdate reagent also served to stop the reaction. Controls for non-enzymic hydrolysis of ATP were made by incubating vesicles separately from the reaction mixture. Controls for ATP hydrolysis by non-specific phosphatase were performed by omitting  $\text{MgSO}_4$  and KCl. As vanadate inhibits non-specific phosphatase as well as plasma membrane ATPase, experiments with vanadate also used a control with vanadate but without  $\text{MgSO}_4$  and KCl.

Pyrophosphatase activity was determined by measuring the release

of inorganic phosphate after a 1 h incubation at 25°C in 0.5 ml of 250 mM glycerol, 50 mM KCl, 0.1 mM PPI-BTP, 5 mM MgSO<sub>4</sub>, 0.25 µg ml<sup>-1</sup> gramicidin D and 20 mM BTP-MES pH 8.0. The assays were performed at pH 8.0 and with 50 mM KCl as the PPase has an alkali pH optimum and is stimulated by K<sup>+</sup> (Walker and Leigh 1981b; Rea and Poole 1985). Phosphate was determined by the method of Bencini et al. (1983) because PPI is rapidly hydrolysed by the Ames acid-molybdate reagent. Addition of the Bencini reagent also served to stop the reaction. Controls for non-enzymic hydrolysis of PPI were made by incubating vesicles separately from the reaction mixture. Controls for PPI hydrolysis by non-specific phosphatase were performed by omitting MgSO<sub>4</sub> and KCl.

Cytochrome c oxidase and antimycin A-insensitive NADH cytochrome c reductase were assayed as described by Hodges and Leonard (1974). Aspartate amino transferase was assayed using an optimized diagnostic kit (Sigma Chemical Co.). Controls with boiled vesicles were used for these assays.

All of the assays used in these experiments gave linear responses with respect to time and protein.

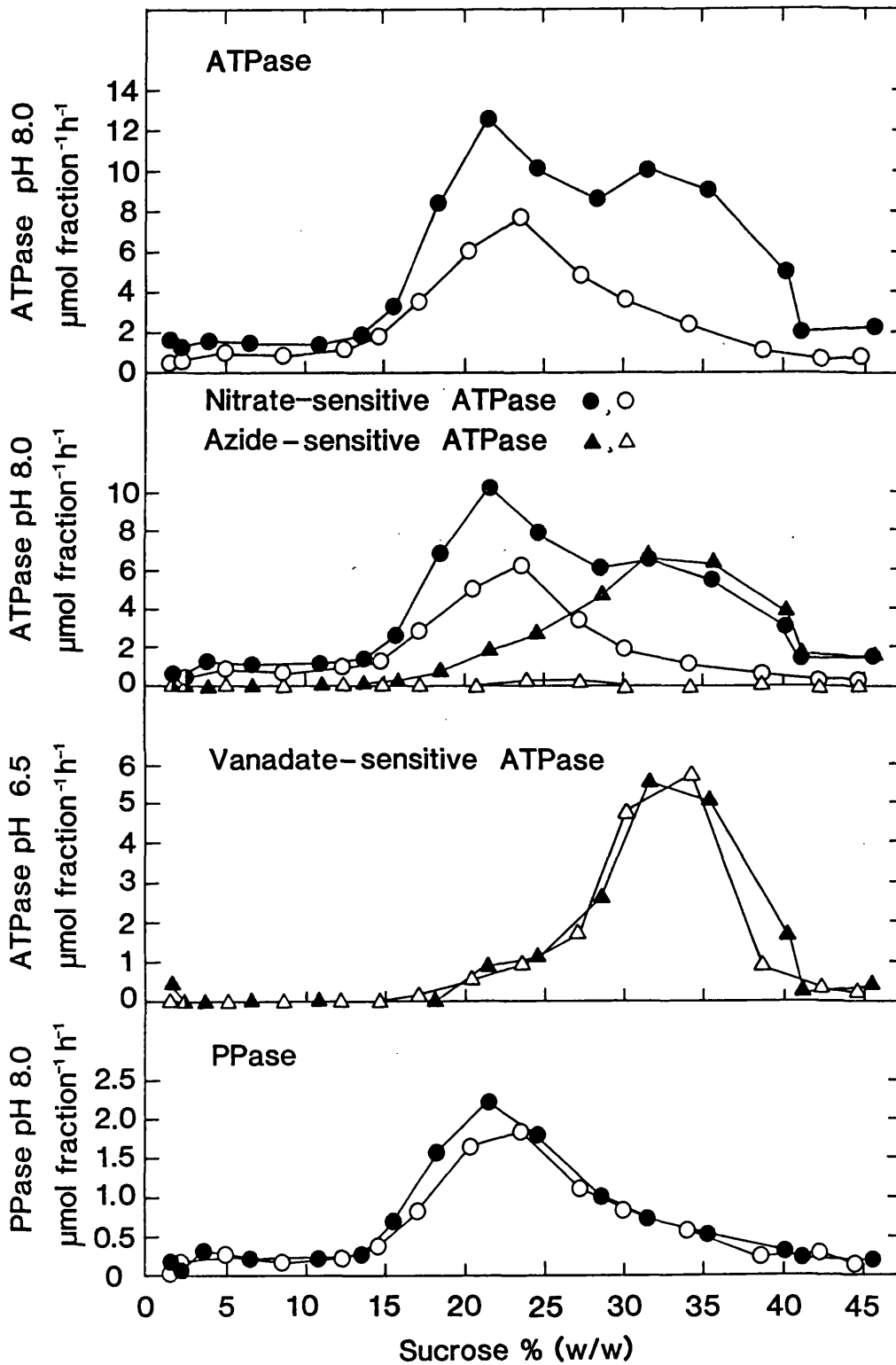
**2.2.4 Protein assay.** A modification of the method of Bradford (1976) was used, with BSA as a standard. The standard method gave an unreliable estimate of membrane protein as the apparent protein concentration in the sample increased as larger volumes were assayed. This was overcome by including 0.003% SDS in the reagent (Appenroth and Augsten 1987) and doubling the dye concentration to maintain sensitivity.

## 2.3 Results

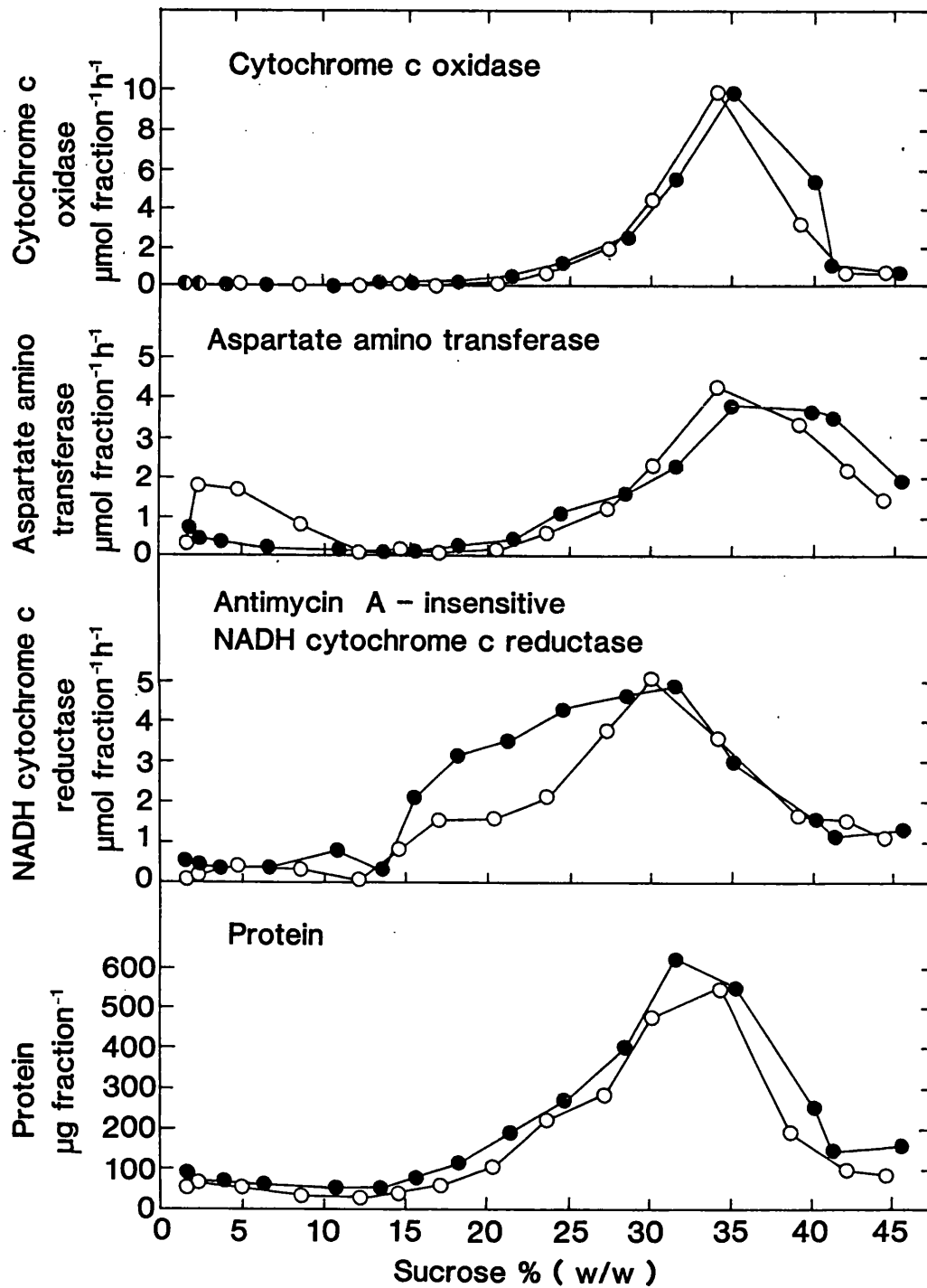
**2.3.1 Fractionation of microsomal membranes on continuous sucrose gradients.** It has been suggested that a wash with 0.25 M KI during the preparation of tonoplast vesicles from sugar beet is necessary to reduce contamination by mitochondrial membrane fragments (Rea et al. 1987a). The KI wash appears to increase the density of membranes having the mitochondrial marker cytochrome c oxidase so that they do not equilibrate with tonoplast on the step gradient (Rea, P.A., Griffith, C.J. and Sanders, D., unpublished data). To find out whether this happens in red beet, microsomal membranes washed in resuspension medium with or without 0.25 M KI were separated on continuous sucrose gradients. The peak densities to which the different marker enzymes sedimented were not affected by KI treatment (Figs. 2.1 and 2.2, Table 2.1).

	Microsomal wash procedure	
	Resuspension medium	KI
	Sucrose concentration in fraction (% [w/w])	
Nitrate-sensitive ATPase (pH 8.0)	22	24
Azide-sensitive ATPase (pH 8.0)	32	-
Vanadate-sensitive ATPase (pH 6.5)	32	34
PPase (pH 8.0)	22	24
Cytochrome c oxidase	35	34
Aspartate amino transferase	35	34
Antimycin A-insensitive NADH cytochrome c reductase	32	30

**Table 2.1.** Effect of 0.25 M KI on the densities to which the peak activities of marker enzymes from washed microsomal membranes sedimented on continuous sucrose gradients. Densities are indicated by the sucrose concentration in the fraction containing peak activity.



**Fig. 2.1.** Distribution of ATPase and PPase activities on continuous sucrose gradients loaded with microsomal membranes washed in either resuspension medium (closed symbols) or 0.25 M KI in resuspension medium (open symbols).



**Fig. 2.2.** Distribution of cytochrome c oxidase, aspartate amino transferase and antimycin A-insensitive NADH cytochrome c reductase activities, and protein on continuous sucrose gradients loaded with microsomal membranes washed in either resuspension medium (closed symbols) or 0.25 M KI in resuspension medium (open symbols).

The recoveries of marker enzyme activities from the sucrose gradients after a resuspension medium wash were generally lower than the activities in the unwashed microsomes (Table 2.2). Measurements were also made of activities in the washed microsomes and the supernatant from the wash step (not shown). These showed that some activity remained in the supernatant and that there was also an actual loss of activity during the wash step. In contrast, the recovery of azide-sensitive ATPase was greatly increased. This may be due to the washing away of an inhibitor of this enzyme in the wash step.

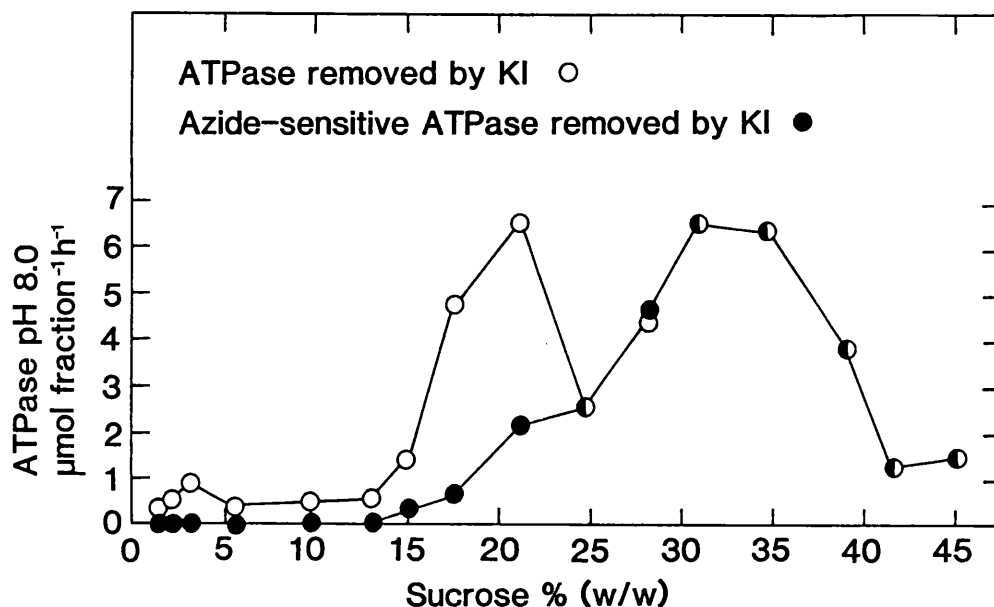
Compared with the resuspension medium wash, the KI wash caused a small loss of the total activities of vanadate-sensitive ATPase (plasma membrane marker), cytochrome c oxidase (mitochondrial marker) and PPase (tonoplast marker) recovered from the gradients and had no effect on the recovery of the plastid marker aspartate

	Microsomal wash procedure		Recovery after KI wash as % of recovery with resuspension medium wash
	Resuspension medium	KI	
	% Recovery		
Nitrate-sensitive ATPase (pH 8.0)	79	36	46
Azide-sensitive ATPase (pH 8.0)	199	4	2
Vanadate-sensitive ATPase (pH 6.5)	89	75	84
PPase (pH 8.0)	57	49	86
Cytochrome c oxidase	56	41	73
Aspartate amino transferase	51	53	104
Antimycin A-insensitive NADH cytochrome c reductase	73	55	75
Protein	84	59	70

**Table 2.2.** Effect of 0.25 M KI on the recovery of marker enzyme activities from continuous sucrose gradients. Recoveries are expressed as a percentage of the activity measured in the unwashed microsomes.



amino transferase (Figs. 2.1 and 2.2, Table 2.2). There was no effect of KI treatment on  $Mg^{2+}$ -independent ATPase measured at pH 6.5, which was less than 10% of the  $Mg^{2+}$ -dependent activity in all fractions (not shown). The  $Mg^{2+}$ -independent activity was used as a measure of non-specific phosphatase, which does not require  $Mg^{2+}$  for activity. However, the KI wash caused a substantial reduction in the recovery of ATPase activity at pH 8.0 and caused a complete loss of azide-sensitive ATPase (mitochondrial ATPase). At densities greater than or equal to that of 25% sucrose loss of ATPase activity can be explained by loss of the mitochondrial ATPase (Fig. 2.3). The loss of azide-sensitive ATPase corresponded exactly to the loss of ATPase activity at these densities. The loss of nitrate-sensitive ATPase in this region (Fig. 2.1) is consistent with this as the mitochondrial ATPase also shows nitrate sensitivity (Grubmeyer and Spencer 1979; Wang and Sze 1985). At lower densities where tonoplast



**Fig. 2.3.** Removal of mitochondrial and tonoplast ATPase activity by 0.25 M KI. The difference in ATPase activity between continuous sucrose gradient fractions from resuspension medium washed microsomes and the corresponding fraction from KI washed microsomes is plotted against fraction sucrose concentration. Where there was a difference in sucrose concentration between two corresponding fractions, an average value was taken. Data taken from Fig. 2.1.

is recovered, the loss of ATPase activity cannot be explained in this way and the KI wash removed 42% of the azide-insensitive ATPase associated with the tonoplast.

The recovery of antimycin A-insensitive NADH cytochrome c reductase was reduced by KI treatment at lower densities but was not affected at densities above that of 30% sucrose. This marker is associated with the ER (Lord et al. 1973). As the membranes were fractionated in the presence of 1 mM EDTA in the experiments described in this chapter, the ER would have been dissociated from its ribosomes, and would have sedimented with a peak at a density of around  $1.12 \text{ g ml}^{-1}$ , equivalent to 29% sucrose (Lord et al. 1973). This would account for the presence of this enzyme in denser fractions. However, this marker is also associated with tonoplast (Leigh and Branton 1976), so the presence of this activity in the fractions of lower density is probably due to tonoplast rather than ER. The KI wash also caused a loss of protein from the lower densities where tonoplast vesicles were recovered.

**2.3.2 Fractionation of microsomal membranes on sucrose step gradients.** To determine the effect of a KI wash on the routine preparation of tonoplast, microsomal membranes were separated on 10/23% (w/w) sucrose step gradients designed to produce a tonoplast-enriched fraction from red beet (Poole et al. 1984). The treatment given to the microsomal suspension greatly affected both the recovery of enzyme activities associated with the tonoplast and the presence of contaminants (Table 2.3). Recoveries of PPase, ATPase and nitrate-sensitive ATPase were largest from resuspension medium washed microsomes. There was negligible azide-sensitive ATPase and cytochrome c oxidase activity, showing that the

the preparations were essentially free of mitochondrial contamination. There was low  $Mg^{2+}$ -independent ATPase activity at pH 6.5, regardless of washing procedure, suggesting that the preparations were substantially free of non-specific phosphatase. Aspartate amino transferase was also low, showing that there was negligible plastid contamination. Both fractions obtained from washed microsomes had no vanadate-sensitive ATPase, indicating that

	Microsomal wash procedure		
	None	Resuspension medium	KI
	Marker enzyme activity		
ATPase (pH 8.0)	10.0 (9)	16.0 (17)	6.1 (14)
ATPase (pH 8.0) (specific activity)	22	65	35
Nitrate-sensitive ATPase (pH 8.0)	7.2 (8)	13.3 (19)	5.3 (12)
Nitrate-sensitive ATPase (pH 8.0) (specific activity)	16	54	30
PPase (pH 8.0)	2.5 (11)	4.1 (22)	2.8 (15)
PPase (pH 8.0) (specific activity)	5.3	17	16
Vanadate-sensitive ATPase (pH 6.5)	2.0 (9)	0 (0)	0 (0)
Azide-sensitive ATPase (pH 8.0)	0.4 (10)	0.3 (6)	0 (0)
Cytochrome c oxidase	1.2 (2)	0.6 (1)	0.5 (1)
$Mg^{2+}$ -independent ATPase (pH 6.5)	0.8 (5)	0.6 (10)	0.6 (10)
Aspartate amino transferase	1.1 (2)	0.5 (1)	0.5 (1)
	Protein		
	0.55 (9)	0.25 (5)	0.17 (5)

**Table 2.3.** Effect of washing procedure on marker enzyme activities and protein in the fraction recovered from a 10/23% (w/w) sucrose step gradient. Enzyme activities are in  $\mu\text{mol h}^{-1}$  and specific activities, where shown, in  $\mu\text{mol mg}^{-1} \text{h}^{-1}$ . Protein recovery in the three fractions is shown in mg. Numbers in parentheses indicate the recovery of activity at the interface of the step gradient, expressed as a percentage of the activity measured in the microsomal suspension after the appropriate wash procedure. Results shown are the average of two preparations.

they were free of plasma membrane contamination. However, if the microsomal wash was omitted, substantial plasma membrane contamination was introduced. The percentage recoveries of the activities present in the microsomal suspensions indicated that this happened because no purification of tonoplast relative to plasma membrane was achieved by the step gradient. There was also a substantial reduction of PPase and nitrate-sensitive ATPase recovered on the step gradient when the microsomal wash was omitted.

The above results indicated that tonoplast vesicles prepared on sucrose step gradients from resuspension medium washed microsomes were purified with respect to contaminating membranes. This is shown more clearly in Table 2.4, where enzyme activities are expressed as specific activities and compared with the specific activities found in the resuspension medium washed microsomal fraction. Specific activities of tonoplast markers were increased 4.5-fold by the sucrose step gradient, whereas specific activities of other markers were decreased.

	Fraction		Purification of marker enzyme by step gradient
	Microsomal	Tonoplast	
Nitrate-sensitive, azide-insensitive ATPase (pH 8.0)	11.5	52	4.5
PPase (pH 8.0)	3.7	16.6	4.5
Vanadate-sensitive ATPase (pH 6.5)	3.4	0	0
Cytochrome c oxidase	11.8	2.4	0.20
Aspartate amino transferase	8.0	2.0	0.25

**Table 2.4.** Specific activities (in  $\mu\text{mol mg}^{-1} \text{h}^{-1}$ ) of marker enzymes from resuspension medium washed microsomes and from tonoplast recovered on a 10/23% (w/w) step gradient from resuspension medium washed microsomes, showing purification of tonoplast markers with respect to other marker enzymes. Results are the average of two preparations.

## 2.4 Discussion

A wash with 0.25 M KI is routinely used during the preparation of tonoplast vesicles from red beet and sugar beet (e.g. Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985; Briskin et al. 1985). The KI wash was originally developed to reduce contamination of plasma membrane preparations from red beet by non-specific phosphatase (Briskin and Poole 1983) and was subsequently used during the preparation of tonoplast (Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985). From the results presented here it can be seen that a KI wash was not necessary to remove  $Mg^{2+}$ -independent ATPase, which was taken as a measure of non-specific phosphatase.

Rea et al. (1987a) suggested that a KI wash was necessary to reduce contamination of tonoplast vesicles by mitochondrial particles in preparations from sugar beet. The results presented in this chapter using cytochrome c oxidase show that this was not necessary when using red beet. Treatment with KI did not alter the density to which mitochondrial membranes sedimented on a continuous sucrose gradient, nor did replacing the KI wash by a resuspension medium wash introduce mitochondrial contamination of tonoplast vesicles prepared on a step gradient. The KI wash did, however, remove mitochondrial ATPase activity from mitochondrial membranes, probably by dissociating the  $F_1$ -ATPase from the membranes. This means that azide-sensitive ATPase cannot be relied upon as a marker for mitochondria in KI washed membranes.

Although using a KI microsomal wash offered no advantages over a resuspension medium wash, omitting a wash step before centrifugation of the membranes on the step gradients led to contamination of the

tonoplast by plasma membrane (Table 2.3). This happened because the step gradient gave no purification of tonoplast markers relative to vanadate-sensitive ATPase. The unwashed microsomal vesicles were clearly behaving very differently on the step gradient. The proper functioning of sucrose gradients to separate membranes depends on the vesicles shrinking osmotically so they separate according to the membrane density rather than the density of their contents (Sze 1985). When the microsomal membranes were not washed, solutes from the brei remaining inside the vesicles may exert an osmotic pressure sufficient to reduce the osmotic shrinkage of the vesicles on the step gradients. This would also explain the reduced recovery of nitrate-sensitive ATPase and PPase if tonoplast vesicles were not sedimenting through the 10% (w/w) sucrose solution. Alternatively, it may be that the wash procedure was necessary to disperse aggregates of vesicles and so enable them to separate properly on the gradients. Aggregates of vesicles containing mainly tonoplast but also some plasma membrane would sediment to the interface of the step gradient, leading to plasma membrane contamination of this fraction. On the other hand, aggregates with a higher proportion of membranes denser than tonoplast would pellet to the bottom of the tube, reducing the recovery of tonoplast markers at the interface of the step gradient. Omitting a microsomal wash also caused a large increase in the amount of protein recovered from the step gradient. This was probably due to both the presence of plasma membrane contamination and to soluble proteins (including the BSA used in the homogenisation medium) normally removed from the vesicles during a microsomal wash.

Comparison of tonoplast vesicles prepared from KI washed microsomes with those from resuspension medium washed microsomes

clearly showed that the KI wash was deleterious to tonoplast. The KI treatment reduced the recovery of the PPase by around 30% and the ATPase by around 60%. Rea et al. (1987a), using tonoplast vesicles prepared from red beet, reported that treatment with 0.24 M KI had no inhibitory effect on ATPase activities subsequently recovered from the step gradient. However, they found similar effects of KI on ATPase activities to those shown here when tonoplast vesicles were prepared from sugar beet (Rea, P.A., Griffith, C.J. and Sanders, D., unpublished data). The reason for these discrepancies is not clear but Rea et al. (1987a) expressed their ATPase activities only as specific activities, which can be misleading as the KI treatment reduces protein content (Fig. 2.2, Table 2.3). In view of the results presented in this chapter it is recommended that tonoplast vesicles should be prepared from resuspension medium washed microsomes rather than from KI washed microsomes. This procedure will be used to prepare the tonoplast vesicles used for the transport studies in the following chapters.

The experiments shown here have taken into account the problems involved in using the mitochondrial ATPase as a marker enzyme. Although previous studies on tonoplast vesicle preparations from red beet did not take this into account (Poole et al. 1984; Bennett et al. 1984; Rea and Poole 1985) the experiments in this chapter show that tonoplast vesicles prepared on a 10/23% sucrose step gradient from washed microsomes are essentially free of contaminating membranes. It is concluded that tonoplast vesicles prepared in this way are therefore suitable for use in studies where transport is studied in response to artificially-imposed gradients.

**CHAPTER 3: THE USE OF ARTIFICIALLY-IMPOSED pH AND Na<sup>+</sup> GRADIENTS TO STUDY Na<sup>+</sup>/H<sup>+</sup> EXCHANGE: ARTEFACTS ASSOCIATED WITH THIS APPROACH**

**3.1 Introduction**

The approach of using artificially-imposed pH and Na<sup>+</sup> gradients to study Na<sup>+</sup>/H<sup>+</sup> antiports in isolated membrane vesicles has been widely applied in a variety of biological systems. As well as providing evidence for the Na<sup>+</sup>/H<sup>+</sup> antiport at the tonoplast in red beet and sugar beet (Blumwald and Poole 1985a; Blumwald and Poole 1987), this approach was used by Katz et al. (1986), who reported a Na<sup>+</sup>/H<sup>+</sup> antiport in plasma membrane vesicles from the halotolerant alga Dunaliella salina. The Na<sup>+</sup>/H<sup>+</sup> antiport observed in many bacterial systems has also been studied in this way (Nakamura et al. 1986). Similarly, imposed gradients have been used extensively to study Na<sup>+</sup>/H<sup>+</sup> antiports in animal tissues (e.g. Murer et al. 1976; Kinsella and Aronson 1980; Warnock et al. 1982; Haigh and Phillips 1989).

Blumwald and Poole (1985a) used a 'pH jump' technique to impose an inside-acid pH gradient on tonoplast vesicles, using the fluorescent probe acridine orange to monitor the pH gradient. They reported that Na salts collapsed the pH gradient. Also, when vesicles loaded with Na<sup>+</sup> were diluted into Na<sup>+</sup>-free medium at the same pH, the Na<sup>+</sup> gradient was able to drive formation of a pH gradient. Furthermore, these effects were still observed in the presence of a K<sup>+</sup>/valinomycin membrane potential clamp, so they could be attributed to the operation of an antiport rather than electrical coupling of fluxes through leak pathways. With a K<sup>+</sup>/



valinomycin clamp,  $\text{Na}^+/\text{H}^+$  exchange showed Michaelis-Menten kinetics. They also reported that the antiport was competitively inhibited by amiloride, an inhibitor of  $\text{Na}^+$  transport in animal systems.

An important assumption in these experiments was that acridine orange fluorescence was correctly reporting changes in the pH gradient. The results presented in this chapter show that this is not always the case. Another assumption was that the  $\text{K}^+$ /valinomycin membrane potential clamp was working correctly. The  $\text{K}^+$ /valinomycin clamp should keep the membrane potential constant in the presence of  $\text{Na}^+$  and not itself transport  $\text{Na}^+$ . The results shown in this chapter also show that these assumptions are not always valid. It is argued from the experiments reported in this chapter that the evidence for a  $\text{Na}^+/\text{H}^+$  antiport at the tonoplast of red beet obtained using pH jumps is artefactual.

## **3.2 Materials and Methods**

**3.2.1 Preparation of tonoplast vesicles.** Tonoplast vesicles were isolated from red beet storage roots as described in section 2.2.2. After removal from the sucrose step gradients, vesicles were loaded with media of desired composition by dilution and centrifugation at 85,000 g ( $r_{\text{av}}$ , Beckman SW 28.1 rotor, 25,000 rpm) for 30 min. The pellets were resuspended in the same media and the vesicles pelleted by centrifugation at 250,000 g ( $r_{\text{av}}$ , Beckman 70.1 Ti rotor, 60,000 rpm) for 10 min. The pellets were resuspended in the same media to give a final protein concentration of 1-3 mg ml<sup>-1</sup>.

### **3.2.2 Measurement of the pH gradient in tonoplast vesicles.**

Inside-acid pH gradients imposed by a pH jump were measured using fluorescent and radioactive probes. The fluorescence of acridine orange (Lee and Forte 1978; Lee et al. 1982) was monitored with a Perkin Elmer LS-5B fluorimeter interfaced to an IBM-XT personal computer. Assay conditions are indicated with the results, but in all experiments tonoplast vesicles were added to 2.0 ml of continuously-stirred buffer at 25°C to give a protein concentration of 7-15 µg ml<sup>-1</sup>. Acridine orange was used at a concentration of 5 µM, with excitation and emission wavelengths of 495 and 540 nm, respectively, and slit widths of 5 nm. Data output from the fluorimeter was handled using software developed by Jennings et al. (1988). Fluorescence was measured in arbitrary units and changes in fluorescence ( $\Delta F/F$ ) were expressed as a percentage of the starting fluorescence. Care was taken in maintaining assay pH and temperature, factors known to affect the reproducibility of results obtained with this technique (Ives et al. 1983).

Uptake of [ $^{14}\text{C}$ ]methylamine (Amersham International plc) was measured at 25°C from a 1.0 ml or 2.0 ml volume containing 20  $\mu\text{M}$  (1.18  $\mu\text{Ci ml}^{-1}$ ) [ $^{14}\text{C}$ ]methylamine. Assay conditions are explained with the results. Uptake was started by the addition of vesicles to give a protein concentration of 15–30  $\mu\text{g ml}^{-1}$ . Aliquots of 100- $\mu\text{l}$  were removed and filtered under vacuum at intervals on Whatman WCN cellulose nitrate membrane filters. The filtration procedure consisted of wetting the filter with 1 ml ice-cold wash medium, rapidly filtering the sample, and rinsing with two 1 ml volumes of ice-cold wash medium. Based on measurements made in the absence of vesicles, the wash procedure removed about 99.7% of the radioactivity on the filter. In the presence of vesicles with an imposed pH gradient of 2 units, the radioactivity accumulated inside the vesicles accounted for about 80% of the radioactivity on the filter. Control experiments were performed to measure pH gradient independent uptake, which was negligible. Uptake of probe that was dependent on the pH gradient was obtained by subtracting the counts obtained in the absence of a pH gradient from those obtained in the presence of an imposed pH gradient. Similarly, uptake of probe that was dependent on the  $\text{Na}^+$  gradient was obtained by subtracting the counts obtained in the absence of a  $\text{Na}^+$  gradient from those obtained in the presence of an imposed  $\text{Na}^+$  gradient. Each treatment was duplicated and each data point is the average of two replicates. The filters were allowed to dry and the radioactivity determined by liquid scintillation counting on a Kontron 'Betamatic' scintillation counter using LKB 'Optiphase RIA' scintillation cocktail. Counts per minute were converted to disintegrations per minute using a channels ratio quench correction curve, which was constructed using acetone as a quenching agent.

In experiments where the membrane potential was clamped with K<sup>+</sup> and valinomycin, vesicles were loaded with a medium containing a K<sup>+</sup> concentration of 40 mM, and the external K<sup>+</sup> concentration required was calculated from the Nernst equation, which at 25°C approximates to:

$$\Delta\Psi = 60 \log([K^+]_o/[K^+]_i)$$

where  $\Delta\Psi$  is in mV and o and i refer to the compartments inside and outside the vesicles.

**3.2.3 Preparation of liposomes.** One g of soybean phosphatidylcholine (Sigma Chemical Co., Type IV-S) was dissolved in 10 ml chloroform and 50- $\mu$ l aliquots were dispensed into vials. The chloroform was removed under a stream of N<sub>2</sub> at room temperature to leave a thin film of phospholipid on the wall of the vial. One ml of N<sub>2</sub>-purged buffer was added, the air in the vial displaced with N<sub>2</sub> and the cap replaced. The vial was then sonicated in an ultrasonic bath in ice-cold water until no further clarification occurred. The liposomes were stored at -20°C and thawed immediately before use.

**3.2.4 Measurement of the pH gradient in liposomes.** Formation and dissipation of pH gradients were measured in liposomes (0.1 mg lipid per assay) using acridine orange fluorescence or [<sup>14</sup>C]methylamine uptake in the same way as described for tonoplast vesicles.

**3.2.5 Measurement of <sup>22</sup>Na uptake in liposomes.** Sodium uptake from a 0.75 ml volume was measured using <sup>22</sup>Na. After addition of liposomes (0.1 mg lipid per assay), an aliquot of labelled

$\text{Na}_2\text{SO}_4$  was added to give  $2.3 \mu\text{Ci ml}^{-1} \text{ }^{22}\text{Na}$ . Subsequent uptake of  $^{22}\text{Na}$  was determined by removing 100- $\mu\text{l}$  aliquots, then filtering, washing and counting as described for [ $^{14}\text{C}$ ]methylamine.

**3.2.6 Protein assay.** Protein was assayed as described in 2.2.4.

**3.2.7 Ionophores.** The  $\text{K}^+$  ionophore valinomycin was made up as a 5 mM stock solution in ethanol. The stock solution was not kept for more than 24 h as it was observed to deteriorate when kept for more than a few days. The ethanol concentration in transport assays resulting from the addition of valinomycin did not exceed 0.1%. Control experiments established that this concentration of ethanol did not affect the assays. The monovalent cation and proton ionophore gramicidin D was made up as a  $0.5 \text{ mg ml}^{-1}$  solution in ethanol, which was stable over many months.

### 3.3 Results

3.3.1 The use of pH jumps to measure  $\text{Na}^+/\text{H}^+$  exchange under the conditions used by Blumwald and Poole (1985a). The pH jump technique was used to impose a 2 unit pH gradient on tonoplast vesicles, without using a  $\text{K}^+$ /valinomycin membrane potential clamp, under the conditions used by Blumwald and Poole (1985a). When vesicles loaded with buffer at pH 6.0 were added to medium at pH 8.0, acridine orange fluorescence was quenched by about 70% (Fig. 3.1). Over the next minute there was a slow and partial recovery of fluorescence. Subsequent addition of  $\text{Na}_2\text{SO}_4$  caused a rapid recovery of fluorescence, as expected if  $\text{Na}^+/\text{H}^+$  exchange was occurring (Blumwald and Poole 1985a). On addition of Triton X-100 to collapse any remaining pH gradient, there was an almost complete recovery of the starting fluorescence.

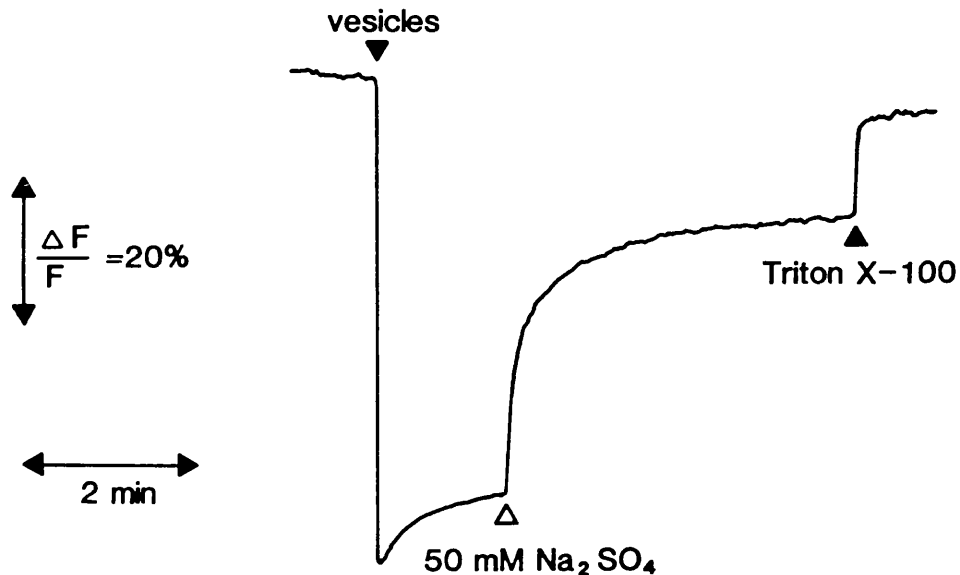
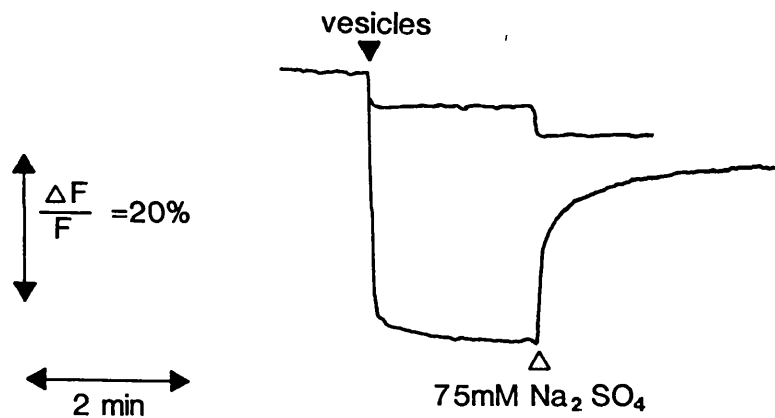


Fig. 3.1. Quenching of acridine orange fluorescence in the presence of a pH gradient, and its recovery by  $\text{Na}_2\text{SO}_4$ . Vesicles loaded with 0.25 M glycerol, 1 mM DTT and 5 mM MES-Tris pH 6.0 were added to a medium containing 0.25 M glycerol, 5  $\mu\text{M}$  acridine orange and 5 mM MES-Tris pH 8.0. Triton X-100 was added as indicated at a concentration of 0.015% (w/v).

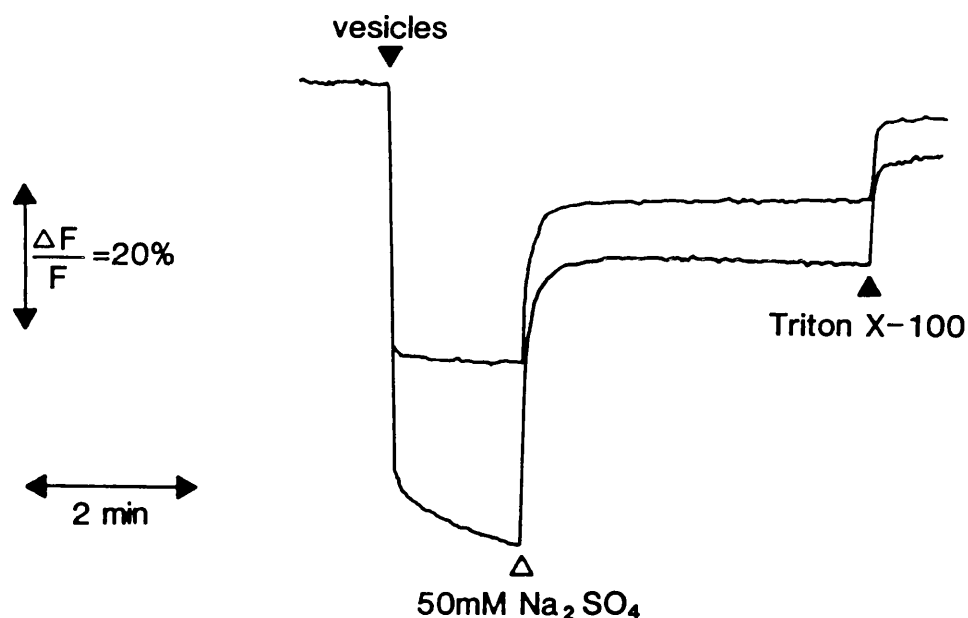
Outwardly-directed  $\text{Na}^+$  gradients were also imposed on tonoplast vesicles in the same way as Blumwald and Poole (1985a). Vesicles were loaded with 75 mM  $\text{Na}_2\text{SO}_4$  at pH 8.0 and diluted 200-fold into medium without  $\text{Na}_2\text{SO}_4$  at pH 8.0. This led to quenching of acridine orange fluorescence (Fig. 3.2), as expected if the  $\text{Na}^+$  gradient was driving pH gradient formation (Blumwald and Poole 1985a). The quenched fluorescence was mostly recovered on addition of  $\text{Na}_2\text{SO}_4$  to a final concentration of 75 mM. When the vesicles were diluted into medium already containing 75 mM  $\text{Na}_2\text{SO}_4$  there was minimal quenching of fluorescence.



**Fig. 3.2.** Quenching of acridine orange fluorescence in the presence of an outwardly-directed  $\text{Na}^+$  gradient and its recovery by  $\text{Na}_2\text{SO}_4$ . Vesicles loaded with 75 mM  $\text{Na}_2\text{SO}_4$ , 0.3 M glycerol, 1 mM DTT and 5 mM MES-Tris pH 8.0 were diluted 200-fold into a medium containing 0.3 M sorbitol, 0.3 M glycerol, 5  $\mu\text{M}$  acridine orange and 5 mM MES-Tris pH 8.0 (lower trace) or containing 75 mM  $\text{Na}_2\text{SO}_4$  instead of 0.3 M sorbitol (upper trace).

The above observations appeared to confirm the presence of the  $\text{Na}^+/\text{H}^+$  antiport in the vesicles used here. However, control experiments were performed to see whether acridine orange was reporting the pH gradient correctly. When vesicles loaded at pH 6.0

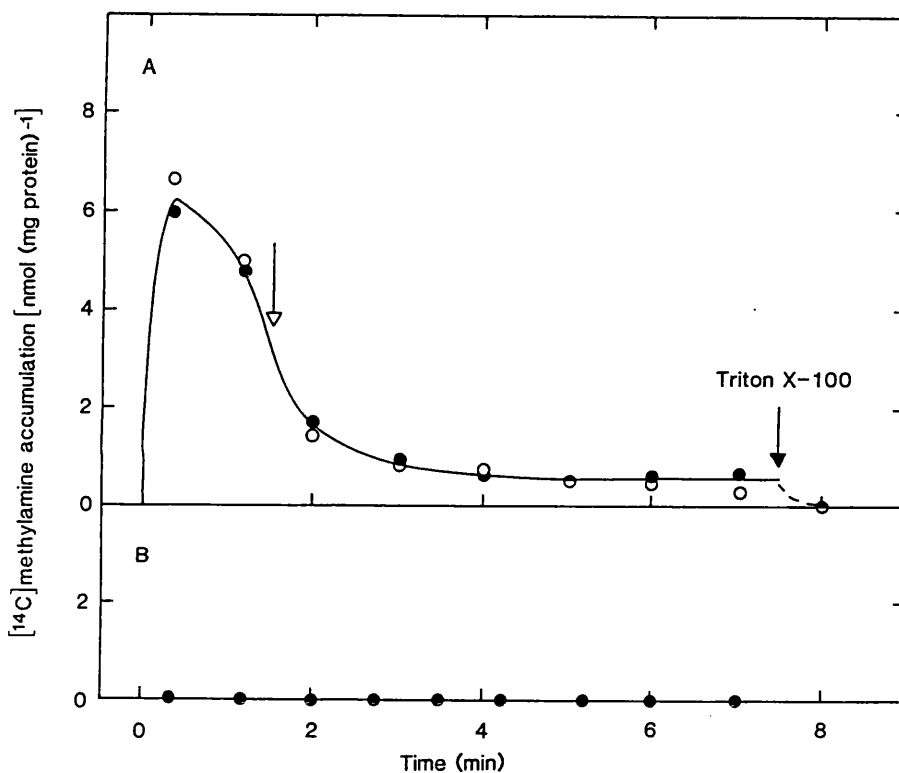
were added to medium at pH 6.0, acridine orange fluorescence was quenched by about 70% and there was a rapid recovery of fluorescence on subsequent addition of Na<sub>2</sub>SO<sub>4</sub> (Fig. 3.3). When the same experiment was performed with intra- and extra-vesicular pH both at pH 8.0, the changes in fluorescence were smaller but followed the same pattern. These controls show that the Na<sup>+</sup>-elicited recovery of fluorescence in the pH jump experiment did not indicate collapse of the pH gradient as this was seen without an imposed pH gradient. Similarly, the quench in acridine orange fluorescence seen on imposition of an outwardly-directed Na<sup>+</sup> gradient did not indicate pH gradient formation, as this quench was seen in the absence of an outwardly-directed Na<sup>+</sup> gradient. This means that the observations in Figs. 3.1 and 3.2 do not confirm the presence of a Na<sup>+</sup>/H<sup>+</sup> antiport.



**Fig. 3.3.** Quenching of acridine orange fluorescence and its reversal by Na<sub>2</sub>SO<sub>4</sub> in the absence of a pH gradient. Vesicles loaded in 0.25 M glycerol, 1 mM DTT and 5 mM MES-Tris at pH 6.0 or 8.0 were added to a medium containing 0.25 M glycerol, 5 μM acridine orange and 5 mM MES-Tris at a pH of either 6.0 or 8.0. In the lower trace both vesicles and media had a pH of 6.0, while in the upper, both had a pH of 8.0. Triton X-100 was added as indicated at a concentration of 0.015% (w/v).



As changes in acridine orange fluorescence appeared to be independent of the pH gradient, [ $^{14}\text{C}$ ]methylamine accumulation was used to measure the pH gradient. Imposition of a 2 unit (inside-acid) pH gradient led to accumulation of the probe (Fig. 3.4A), showing that [ $^{14}\text{C}$ ]methylamine accumulation was dependent on the presence of a pH gradient. However, the [ $^{14}\text{C}$ ]methylamine was lost rapidly and this was independent of the  $\text{Na}^+$  treatment imposed.



**Fig. 3.4A,B.** Accumulation of [ $^{14}\text{C}$ ]methylamine by tonoplast vesicles dependent upon a 2 unit pH gradient (A) or dependent upon a 200-fold outwardly-directed  $\text{Na}^+$  gradient (B). In (A), vesicles were loaded with 0.25 M glycerol, 1 mM DTT and 5 mM MES-Tris pH 6.0 and added to 2.0 ml of 0.25 M glycerol, 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]methylamine and 5 mM MES-Tris pH 8.0 at  $t=0$ . Samples of 100- $\mu\text{l}$  were removed at intervals and the vesicles filtered and washed as described in 3.2.2. The wash medium used consisted of 0.25 M glycerol and 5 mM MES-Tris pH 8.0. As indicated by ( $\downarrow$ ),  $\text{Na}_2\text{SO}_4$  was added to a concentration of 50 mM in the treatment marked by (●) or 0 mM in the treatment marked by (○). Triton X-100 was added as indicated at a concentration of 0.015% (w/v) to dissipate any residual pH gradient. In (B), vesicles were loaded with 75 mM  $\text{Na}_2\text{SO}_4$ , 0.3 M glycerol, 1 mM DTT and 5 mM MES-Tris pH 8.0 and diluted 200-fold into 1.0 ml 0.3 M sorbitol, 0.3 M glycerol, 20  $\mu\text{M}$  [ $^{14}\text{C}$ ]methylamine and 5 mM MES-Tris pH 8.0 at  $t=0$ . Samples of 100- $\mu\text{l}$  were removed at intervals and filtered and washed as described above.

When an outwardly-directed  $\text{Na}^+$  gradient was imposed on tonoplast vesicles using the same conditions as in Fig. 3.2, except that [ $^{14}\text{C}$ ]methylamine was used as the pH gradient probe instead of acridine orange, there was no measurable  $\text{Na}^+$  gradient dependent [ $^{14}\text{C}$ ]methylamine accumulation (Fig. 3.4B). This shows that the  $\text{Na}^+$  gradient was not able to generate a pH gradient.

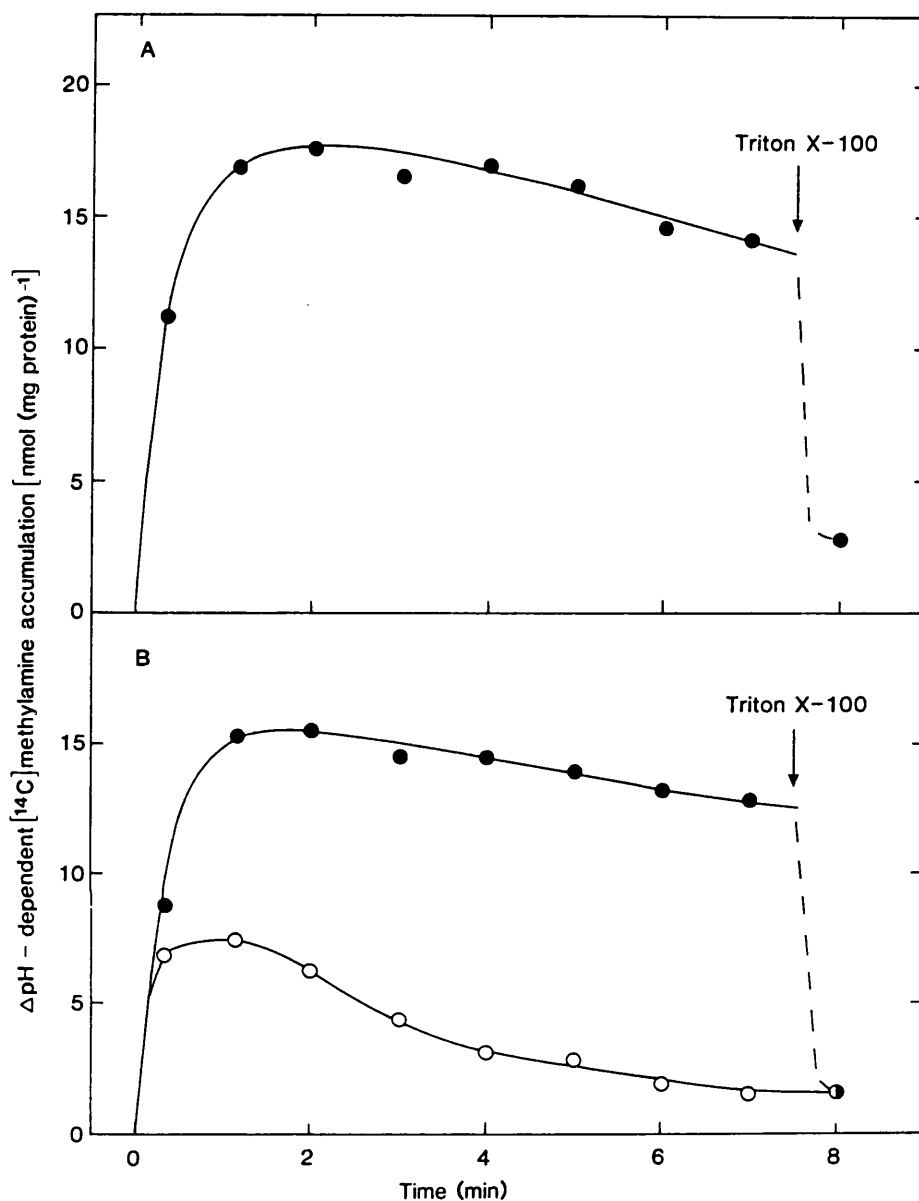
The observations made with [ $^{14}\text{C}$ ]methylamine do not provide any evidence for the presence of a  $\text{Na}^+/\text{H}^+$  antiport. However, it is possible that this may be due to the instability of the pH gradient under these conditions (Fig. 3.4A), rather than to the absence of this transport system.

**3.3.2 Development of the pH jump technique for measuring  $\text{Na}^+/\text{H}^+$  exchange.** It was found that the above problems with the pH jump technique were dependent on media composition and could be avoided by using media of a different composition. The compositions of the media used for loading the vesicles and the media to which the vesicles were added in a pH jump were changed for the reasons outlined below. Vesicles were loaded at pH 5.5 and a pH jump imposed by adding to medium at pH 7.5 as these pH values are closer to the vacuolar and cytoplasmic pH values respectively. BTP buffers were used instead of Tris buffers as it has been reported that Tris buffers are uncoupling (Good et al. 1966; Sone et al. 1977). The pH of BTP buffer systems was also found to be less susceptible to salt effects than Tris buffer systems. Addition of 50 mM  $\text{Na}_2\text{SO}_4$  to the pH 7.5 medium in the absence of vesicles had little effect on the pH, causing an increase of about 0.05 units. As a precaution against osmotically-induced vesicle volume changes affecting the results, sorbitol was used instead of glycerol as an osmoticum

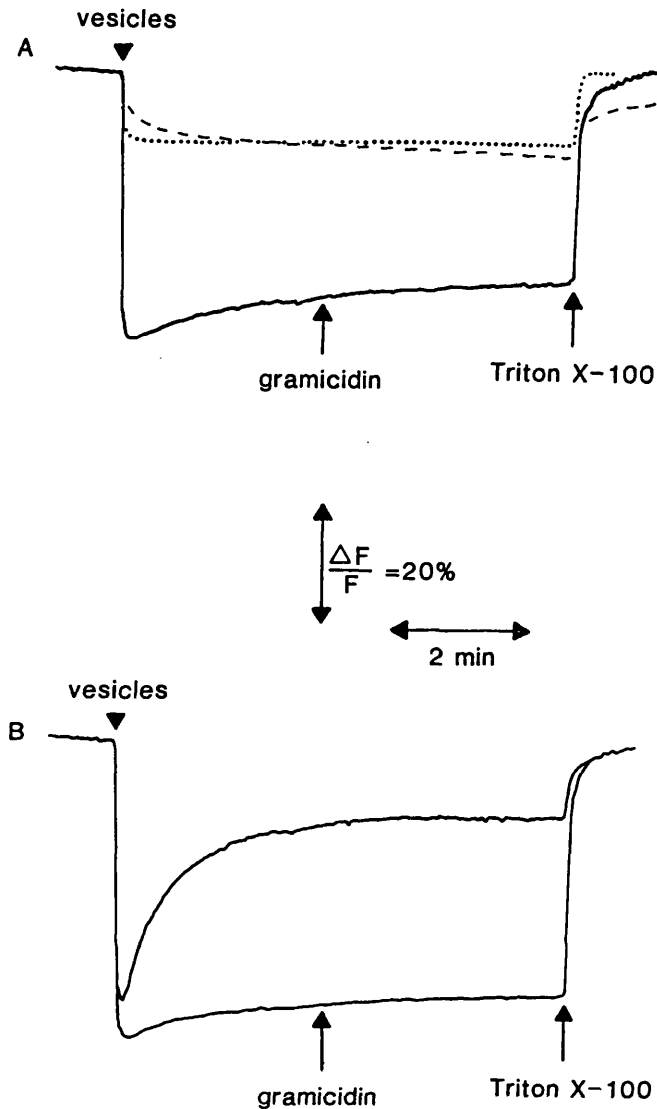
as glycerol is too permeable to provide any osmotic buffering in red beet tonoplast vesicles (Pope and Leigh 1988b). In addition, where the effect of adding different concentrations of  $\text{Na}_2\text{SO}_4$  was investigated, dilutions of the  $\text{Na}_2\text{SO}_4$  stock were made with a sorbitol stock solution so that all additions caused the same change in osmolarity. EGTA was included in the pH 7.5 medium to chelate any contaminating  $\text{Ca}^{2+}$ , which could collapse the pH gradient via the  $\text{Ca}^{2+}/\text{H}^+$  antiport (Blumwald and Poole 1986).

When a pH jump was used to impose a 2 unit gradient on tonoplast vesicles under these conditions, in the absence of a  $\text{K}^+$ /valinomycin membrane potential clamp, accumulation of [ $^{14}\text{C}$ ]methylamine indicated that the pH gradient was stable (Fig. 3.5A). However, when a 2 unit pH gradient was imposed on tonoplast vesicles clamped at 0 mV with  $\text{K}^+$  and valinomycin, the pH gradient was unstable (Fig. 3.5B). Therefore, the procedure of Rea et al. (1987b) was used, in which the membrane potential was clamped at -120 mV, the equilibrium potential for  $\text{H}^+$  for a 2 unit pH gradient at 25°C. Under these conditions, both the extent and time course of [ $^{14}\text{C}$ ]methylamine accumulation were similar to that found without a clamp, showing that the pH gradient was stable (Fig. 3.5B).

When pH jump experiments were performed under the same set of conditions used in Fig. 3.5, but using acridine orange to monitor the pH gradient, the way acridine orange fluorescence reported the pH gradient was found to be in agreement with the results with [ $^{14}\text{C}$ ]methylamine (Fig. 3.6). When tonoplast vesicles loaded at pH 5.5 were added to medium at pH 7.5 the fluorescence of acridine orange was quenched by about 50% and the signal was stable. When the vesicles were disrupted with Triton X-100, most of the fluorescence



**Fig. 3.5A,B.** Accumulation of [ $^{14}$ C]methylamine by tonoplast vesicles in response to a 2 unit pH gradient, without a membrane potential clamp (A) or clamped at 0 (B,  $\circ$ ) or  $-120$  mV (B,  $\bullet$ ). In (A) vesicles were loaded with 0.55 M sorbitol, 1 mM EDTA, 2 mM DTT and 5 mM BTP-MES pH 5.5 and diluted into 1.0 ml of 0.47 M sorbitol, 0.3 mM EGTA, 20  $\mu$ M [ $^{14}$ C]methylamine and 10 mM BTP-HEPES pH 7.5. In (B) vesicles were loaded with 0.5 M sorbitol, 20 mM  $K_2SO_4$ , 1 mM EDTA, 2 mM DTT and 5 mM BTP-MES pH 5.5 and diluted into 1.0 ml 0.47 M sorbitol, 0.3 mM EGTA, 20  $\mu$ M [ $^{14}$ C]methylamine, 5  $\mu$ M valinomycin, 10 mM BTP-HEPES pH 7.5 with an external  $K_2SO_4$  concentration of either 20 mM ( $\circ$ , to clamp at 0 mV), or 0.2 mM ( $\bullet$ , to clamp at  $-120$  mV). Samples of 100- $\mu$ l were removed at intervals and filtered and washed as described in 3.2.2. The wash medium contained 0.47 M sorbitol, 0.3 mM EGTA and 10 mM BTP-HEPES pH 7.5. Triton X-100 was added as indicated to a concentration of 0.015% to collapse pH gradients.



**Fig. 3.6A,B.** Quenching of acridine orange fluorescence in response to a 2 unit pH gradient in tonoplast vesicles, without a membrane potential clamp (A, —) or clamped at 0 (B, upper trace) or  $-120$  mV (B, lower trace), showing smaller quenching of fluorescence without a pH gradient at pH 5.5 (A, ---) or 7.5 (A, .....). In (A) vesicles were loaded with 0.55 M sorbitol, 1 mM EDTA, 2 mM DTT and 5 mM BTP-MES pH 5.5 and diluted into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu$ M acridine orange, 10 mM BTP-HEPES pH 7.5 (—), or diluted into 0.55 M sorbitol, 1 mM EDTA, 5  $\mu$ M acridine orange, 5 mM BTP-MES pH 5.5 (---). Vesicles were also loaded with 0.47 M sorbitol, 0.3 mM EGTA, 2 mM DTT and 10 mM BTP-HEPES pH 7.5 and diluted into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu$ M acridine orange and 10 mM BTP-HEPES pH 7.5 (.....). In (B) vesicles were loaded with 0.5 M sorbitol, 20 mM  $K_2SO_4$ , 1 mM EDTA, 2 mM DTT and 5 mM BTP-MES pH 5.5 and diluted into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu$ M acridine orange, 5  $\mu$ M valinomycin, 10 mM BTP-HEPES pH 7.5 with an external  $K_2SO_4$  concentration of either 20 mM (to clamp at 0 mV, upper trace), or 0.2 mM (to clamp at  $-120$  mV, lower trace). Gramicidin was added as indicated to a concentration of 0.5  $\mu$ g ml $^{-1}$ . Triton X-100 was added as indicated at a concentration of 0.015% (w/v).

signal was recovered. The addition of gramicidin did not have any effect on the pH gradient. In control experiments, where vesicles were added to medium at pH 5.5 or vesicles loaded at pH 7.5 were added to medium at pH 7.5, there was only a small quench in fluorescence. When the size of the imposed pH jump was varied by changing the pH of the medium to which the vesicles were added, the probe showed greater sensitivity as the size of the pH gradient increased (Fig. 3.7). A log plot gave a straight line (Fig. 3.7 inset), as found by Lee and Forte (1978).

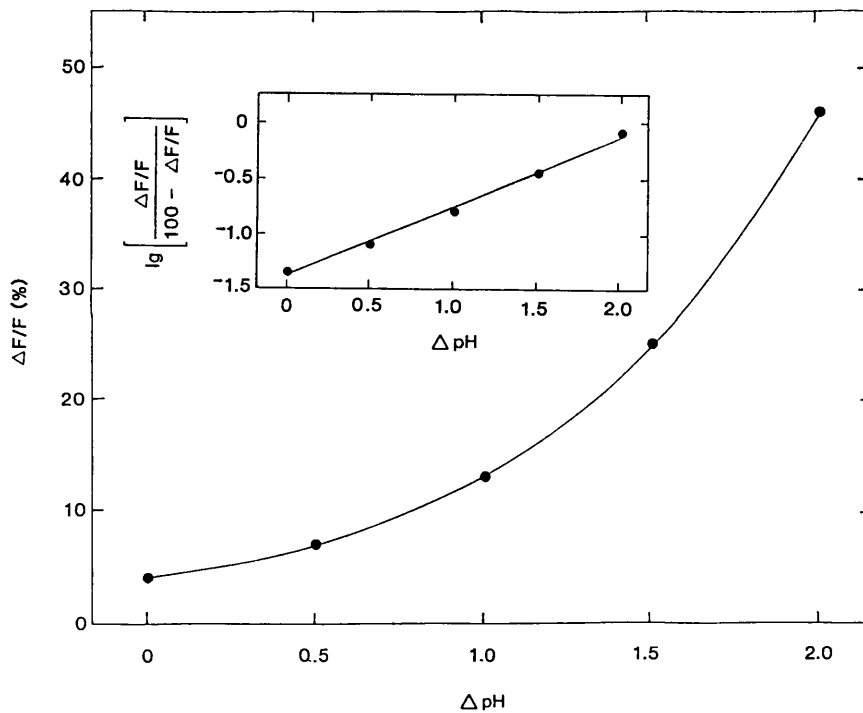
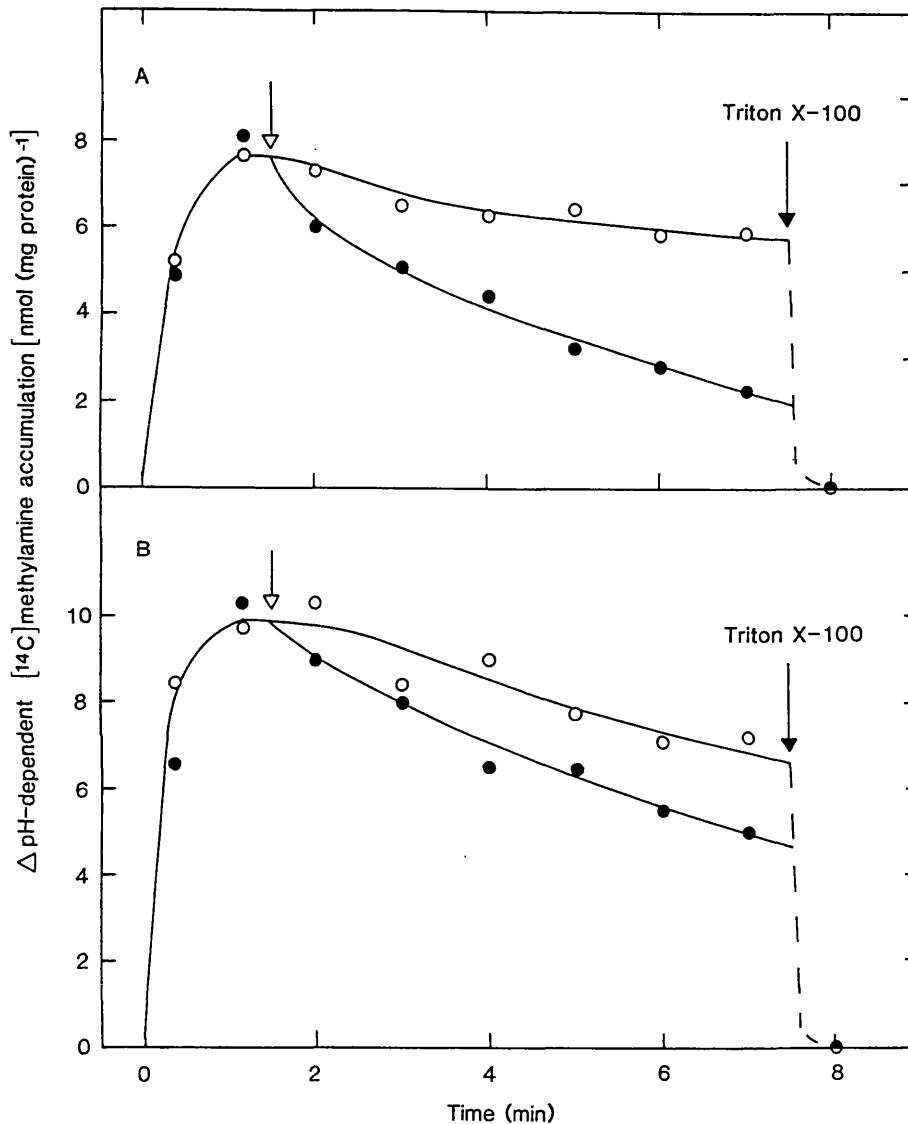


Fig. 3.7. Quench of acridine orange fluorescence against imposed pH gradient and (inset) log transformation of the data.

When a 2 unit pH gradient was imposed on tonoplast vesicles clamped at 0 mV, the fluorescence quench was smaller than without a clamp and the quenched fluorescence was quickly recovered, showing that the pH gradient was unstable (Fig. 3.6B). At -120 mV the fluorescence was quenched by about 50% and the signal then stayed

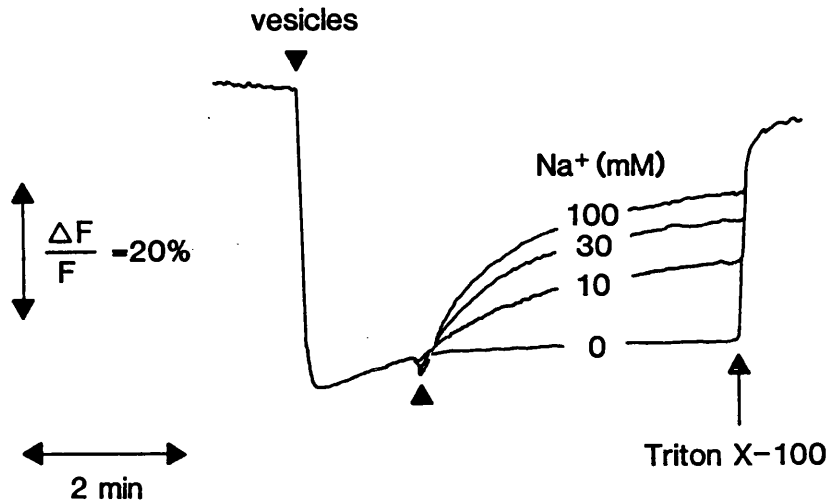
fairly constant, showing that the gradient was stable. The addition of gramicidin had no effect on the stability of the pH gradient at -120 mV. Further experiments were carried out using the -120 mV clamp as the 0 mV clamp was clearly unsatisfactory.

When a 2 unit pH gradient was imposed on tonoplast vesicles clamped at -120 mV, accumulation of [ $^{14}\text{C}$ ]methylamine reported that the pH gradient was dissipated by  $\text{Na}_2\text{SO}_4$  (Fig. 3.8A), as expected if  $\text{Na}^+/\text{H}^+$  exchange was occurring. Surprisingly, when valinomycin was omitted, the extent of the  $\text{Na}^+$ -induced collapse of the pH gradient was smaller (Fig. 3.8B), suggesting that  $\text{Na}^+/\text{H}^+$  exchange was electrogenic. When acridine orange was used as the pH probe under the same set of conditions, a collapse of the pH gradient by  $\text{Na}_2\text{SO}_4$  was also reported (Fig. 3.9). These results, together with those shown in Figs. 3.5 and 3.6, indicated that acridine orange fluorescence was a reliable reporter of the pH gradient under these conditions. The quenching of acridine orange fluorescence was dependent on the pH gradient and the results obtained were in agreement with those obtained with [ $^{14}\text{C}$ ]methylamine. As monitoring acridine orange fluorescence gives better time resolution than measuring uptake of [ $^{14}\text{C}$ ]methylamine, acridine orange fluorescence was used routinely for measurements of the pH gradient. The initial rate of collapse of the pH gradient, measured with acridine orange fluorescence, showed Michaelis-Menten kinetics (Fig. 3.10) and Hanes-Woolf plots gave a  $K_m$  for  $\text{Na}^+$  of  $51.3 \pm 4.9$  mM and a  $V_{\text{max}}$  of  $43.4 \pm 5.1$  %F  $\text{min}^{-1}$  (average  $\pm$  SE, seven tonoplast preparations). These results are consistent with the presence of a saturable  $\text{Na}^+/\text{H}^+$  antiport, as the procedure of clamping the membrane potential should eliminate any electrical coupling of  $\text{Na}^+$  and  $\text{H}^+$  fluxes.

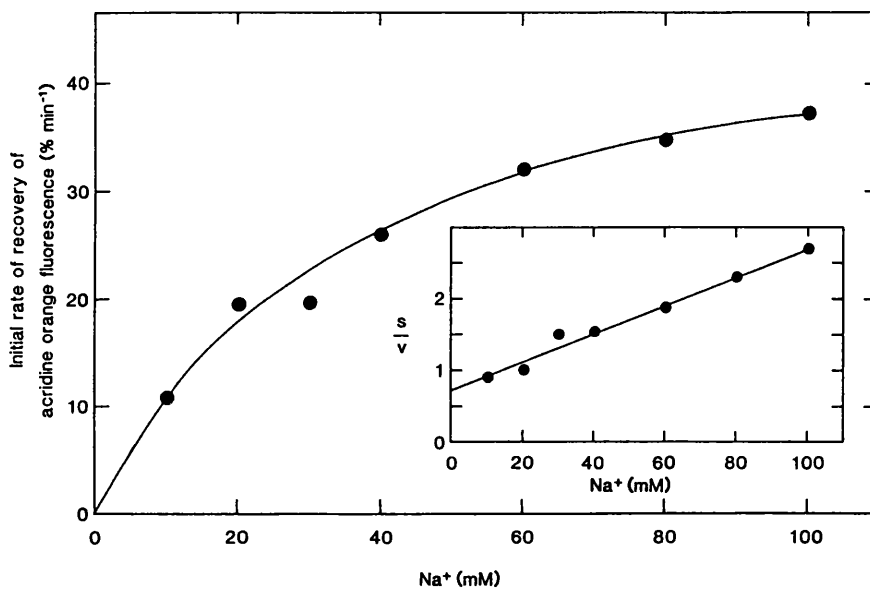


**Fig. 3.8A,B.** Collapse of pH gradient by  $\text{Na}_2\text{SO}_4$  in tonoplast vesicles either clamped at  $-120$  mV with  $\text{K}^+$ /valinomycin (A), or without valinomycin (B), measured by  $[^{14}\text{C}]$ methylamine uptake. Vesicles were loaded with  $0.5$  M sorbitol,  $20$  mM  $\text{K}_2\text{SO}_4$ ,  $1$  mM EDTA,  $2$  mM DTT and  $5$  mM BTP-MES pH  $5.5$  and diluted into  $1.0$  ml  $0.47$  M sorbitol,  $0.3$  mM EGTA,  $20$   $\mu\text{M}$   $[^{14}\text{C}]$ methylamine,  $10$  mM BTP-HEPES pH  $7.5$  to give an external  $\text{K}_2\text{SO}_4$  concentration of  $0.2$  mM, either with (A) or without (B)  $5$   $\mu\text{M}$  valinomycin. Samples of  $100$ - $\mu\text{l}$  were removed at intervals and filtered and washed as described in 3.2.2. At the indicated time ( $\downarrow$ ), an aliquot of  $\text{Na}_2\text{SO}_4$  or sorbitol was added to give a  $\text{Na}^+$  concentration of  $30$  mM ( $\bullet$ ) or  $0$  mM ( $\circ$ ). Triton X-100 was added as indicated at a concentration of  $0.015\%$  (w/v).





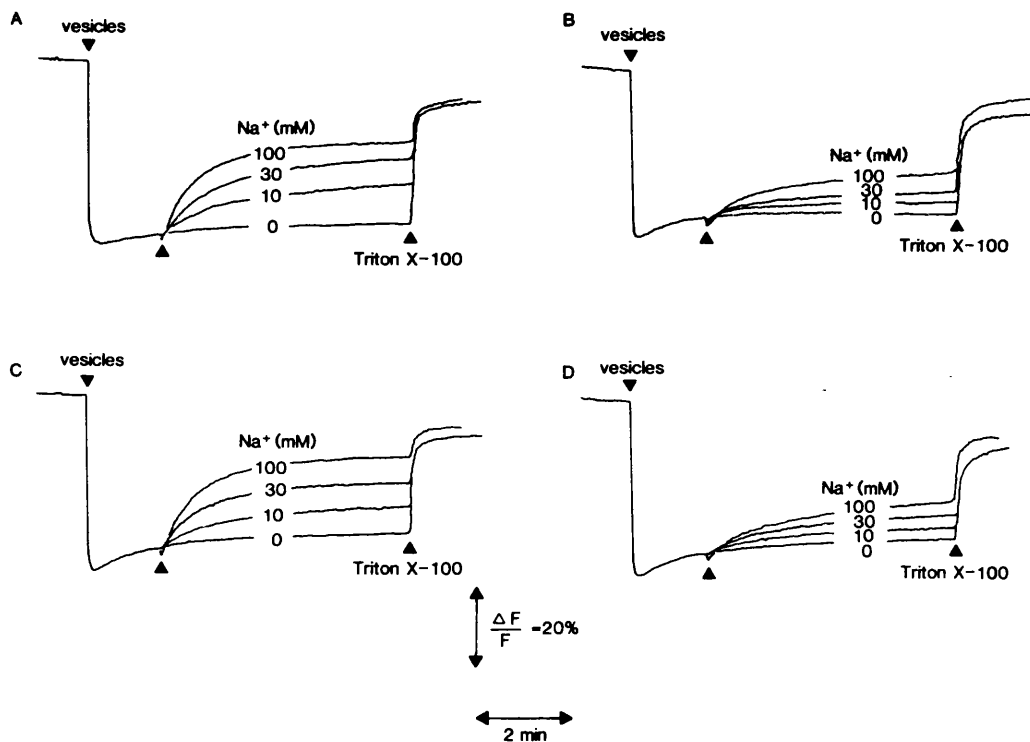
**Fig. 3.9.** Collapse of pH gradient by  $\text{Na}_2\text{SO}_4$  in tonoplast vesicles clamped at  $-120$  mV, measured by acridine orange fluorescence. Vesicles were loaded with  $0.5$  M sorbitol,  $20$  mM  $\text{K}_2\text{SO}_4$ ,  $1$  mM EDTA,  $2$  mM DTT and  $5$  mM BTP-MES pH  $5.5$  and diluted into a medium containing  $0.47$  M sorbitol,  $0.3$  mM EGTA,  $5$   $\mu\text{M}$  acridine orange,  $5$   $\mu\text{M}$  valinomycin,  $10$  mM BTP-HEPES pH  $7.5$  to give an external  $\text{K}_2\text{SO}_4$  concentration of  $0.2$  mM. Addition of  $\text{Na}_2\text{SO}_4$  or sorbitol to give the indicated  $\text{Na}^+$  ion concentration is shown by ( $\blacktriangle$ ). Triton X-100 was added as indicated at a concentration of  $0.015\%$  (w/v).



**Fig. 3.10A,B.** Concentration dependence of the initial rate of  $\text{Na}_2\text{SO}_4$ -induced collapse of the pH gradient imposed by a pH jump in tonoplast vesicles clamped at  $-120$  mV. Inset: Hanes-Woolf plot of the data.

### 3.3.3 The role of valinomycin and the lipid bilayer in $\text{Na}^+/\text{H}^+$

exchange. An important assumption in the use of a  $\text{K}^+$ /valinomycin clamp is that the clamp only serves to keep the membrane potential constant during the experiment, and does not have any other effect on the system under study. This assumption was tested by performing pH jumps with and without valinomycin and  $\text{K}^+$  (Fig. 3.11). It was found that the rate and extent of the  $\text{Na}^+$ -induced collapse of the pH gradient were much lower when valinomycin was omitted in both the presence and absence of  $\text{K}^+$  (Figs. 3.11, 3.12). The initial rate of



**Fig. 3.11A,B,C,D.** Collapse of pH gradients by  $\text{Na}_2\text{SO}_4$  in tonoplast vesicles clamped at  $-120$  mV with  $\text{K}^+$ /valinomycin (A), with  $\text{K}^+$  but without valinomycin (B), without  $\text{K}^+$  but with valinomycin (C), or without  $\text{K}^+$  and without valinomycin (D). Vesicles were loaded with 0.5 M sorbitol, 1 mM EDTA, 2 mM DTT and 5 mM BTP-MES pH 5.5 with (A,B) or without (C,D) 20 mM  $\text{K}_2\text{SO}_4$ , and diluted 100-fold into a medium containing 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu\text{M}$  acridine orange and 10 mM BTP-HEPES pH 7.5 either with (A,C) or without (B,D) 5  $\mu\text{M}$  valinomycin. Sodium sulphate or sorbitol was added at ( $\blacktriangle$ ) to give the indicated  $\text{Na}^+$  concentration. Triton X-100 was added as indicated at a concentration of 0.015% (w/v).

collapse of the pH gradient was dependent upon the  $\text{Na}^+$  concentration and the presence of valinomycin but was independent of  $\text{K}^+$  (Fig. 3.12). Instead of simply clamping the membrane potential through a  $\text{K}^+$  conductance, valinomycin was itself increasing the rate of  $\text{Na}^+/\text{H}^+$  exchange.

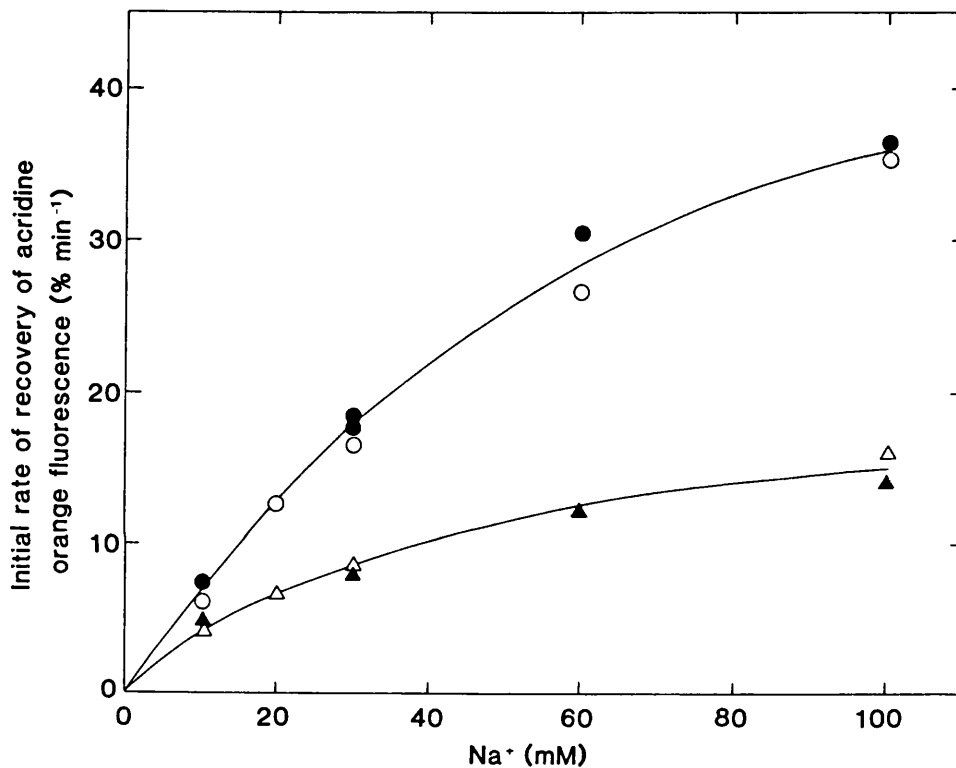


Fig. 3.12. Concentration dependence of the initial rate of  $\text{Na}_2\text{SO}_4$ -induced collapse of the pH gradient imposed by a pH jump with  $\text{K}^+$ /valinomycin (●), with  $\text{K}^+$  but without valinomycin (▲), without  $\text{K}^+$  but with valinomycin (○), or without  $\text{K}^+$  and without valinomycin (△). Data taken from Fig. 3.11.

In order to determine whether the effect of valinomycin was of any physiological significance, pH jump experiments were performed using phosphatidylcholine liposomes instead of tonoplast vesicles. When a 2 unit pH gradient was imposed on liposomes in the absence of a  $\text{K}^+$ /valinomycin clamp, the fluorescence of acridine orange was

quenched by about 50% (Fig. 3.13A) and a stable fluorescence signal was obtained. The addition of gramicidin did not have any effect on the pH gradient. In the absence of a pH gradient at pH 7.5 or 5.5 there was only a very small quench in fluorescence on addition of liposomes, showing that acridine orange fluorescence was responding to the pH gradient. As found in tonoplast vesicles, a 2 unit pH gradient imposed by a pH jump was unstable when clamped at 0 mV (Fig. 3.13B). At -120 mV the pH gradient was stable and the addition of gramicidin was without effect.

A 2 unit pH gradient imposed on liposomes in the presence of a -120 mV clamp was dissipated on addition of  $\text{Na}_2\text{SO}_4$  (Fig. 3.14A). The initial rate of collapse of the pH gradient showed saturating kinetics (Fig. 3.15) and Hanes-Woolf plots gave a  $K_m$  for  $\text{Na}^+$  of  $28.5 \pm 4.0$  mM and a  $V_{max}$  of  $30.0 \pm 5.8$  %F  $\text{min}^{-1}$  (average  $\pm$  SE, five liposome preparations). The pH gradient was collapsed by Na-IDA but not by sulphate-BTP, showing that  $\text{Na}^+$  was responsible for the collapse of the pH gradient (Fig. 3.14B). In the absence of valinomycin, both the initial rate and the extent of collapse of the pH gradient were much lower on addition of  $\text{Na}_2\text{SO}_4$  (Fig. 3.14C). In the presence of 100 mM  $\text{Na}^+$ , the initial rate of recovery of acridine orange fluorescence was 5 %F  $\text{min}^{-1}$ .

These results show that the ability of valinomycin to increase the rate of  $\text{Na}^+$ -induced collapse of the pH gradient in tonoplast vesicles was due to a general effect on the membrane. Valinomycin appeared to increase the rate of  $\text{Na}^+/\text{H}^+$  exchange through the bilayer. The results obtained with liposomes also show that Michaelis-Menten kinetics do not necessarily indicate that a physiological transporter is involved.

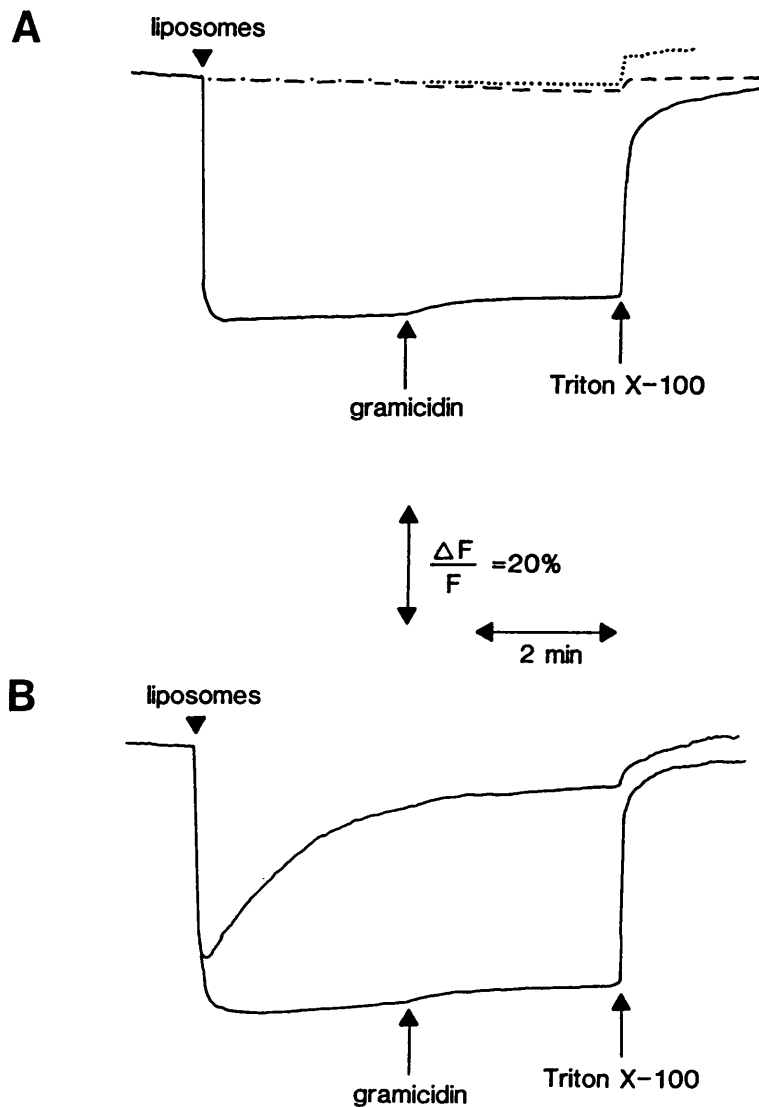
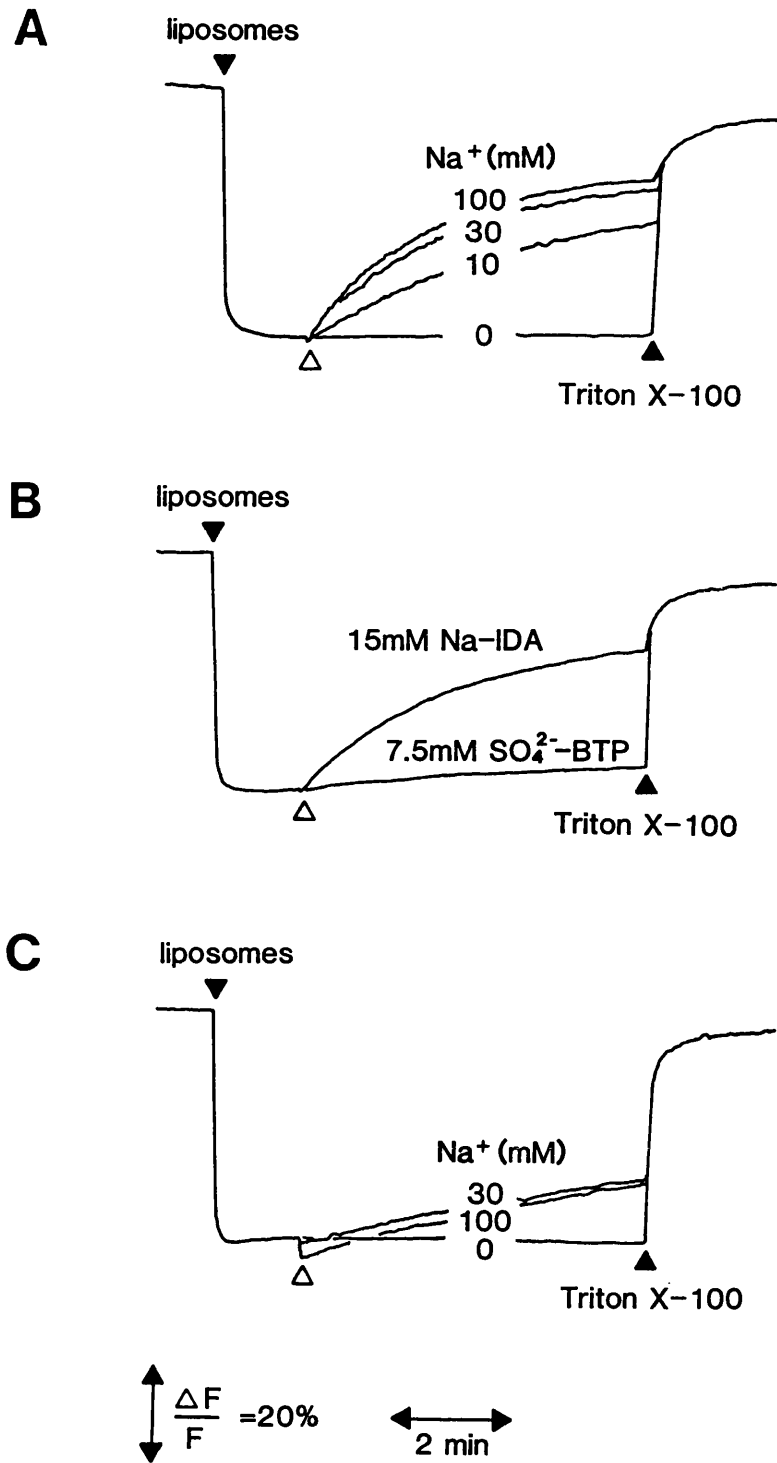
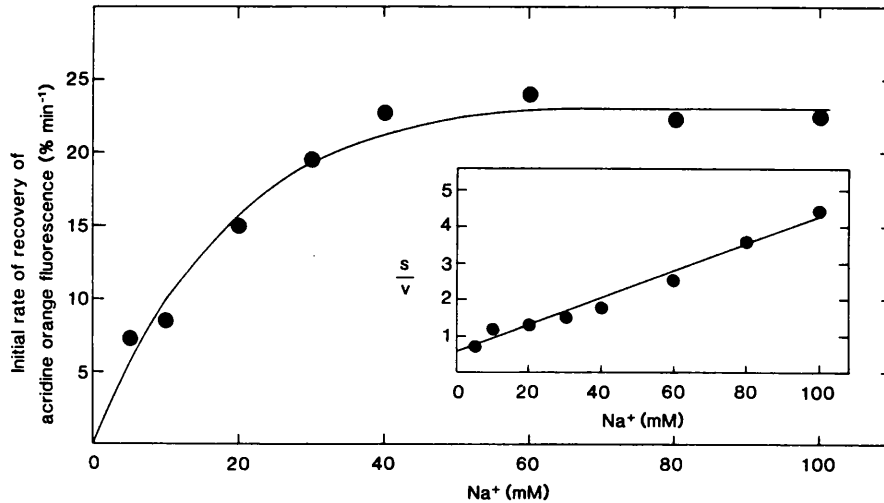


Fig. 3.13A,B. Quenching of acridine orange fluorescence in response to a 2 unit pH gradient in liposomes, without a membrane potential clamp (A) or clamped at 0 (B, upper trace) or  $-120$  mV (B, lower trace), showing no quenching of fluorescence in the absence of a pH gradient at pH 5.5 (A, - - -) or pH 7.5 (A, ·····). In (A) liposomes were loaded with 0.55 M sorbitol, 1 mM EDTA, and 5 mM BTP-MES pH 5.5 and diluted into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu$ M acridine orange, 10 mM BTP-HEPES pH 7.5 (—), or 0.55 M sorbitol, 1 mM EDTA, 5  $\mu$ M acridine orange, 5 mM BTP-MES pH 5.5 (- - -). Liposomes were also loaded with 0.47 M sorbitol, 0.3 mM EGTA, and 10 mM BTP-HEPES pH 7.5 and diluted into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu$ M acridine orange and 10 mM BTP-HEPES pH 7.5 (·····). In (B) liposomes were loaded with 0.5 M sorbitol, 20 mM  $K_2SO_4$ , 1mM EDTA, and 5 mM BTP-MES pH 5.5 and diluted into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu$ M acridine orange, 5  $\mu$ M valinomycin, 10 mM BTP-HEPES pH 7.5 with an external  $K_2SO_4$  concentration of either 20 mM (to clamp at 0 mV, upper trace) or 0.2 mM (to clamp at  $-120$  mV, lower trace). Gramicidin was added as indicated to a concentration of 0.5  $\mu$ g ml $^{-1}$ . Triton X-100 was added as indicated at a concentration of 0.015% (w/v).



**Fig. 3.14A,B,C.** Collapse of pH gradient by Na salts in liposomes. Liposomes were prepared in 0.5 M sorbitol, 20 mM K<sub>2</sub>SO<sub>4</sub>, 1 mM EDTA, 5 mM BTP-MES pH 5.5 and diluted 100-fold into 0.47 M sorbitol, 0.3 mM EGTA, 5 μM acridine orange, 10 mM BTP-HEPES pH 7.5, with (A,B) or without (C) 5 μM valinomycin. In (A) and (C) addition of Na<sub>2</sub>SO<sub>4</sub> or sorbitol to give the indicated Na<sup>+</sup> ion concentration is shown by (Δ). In (B) Na-IDA or SO<sub>4</sub>-BTP were added at (Δ) as indicated. Triton X-100 was added as indicated at a concentration of 0.015% (w/v) to collapse any residual pH gradient.



**Fig. 3.15.** Concentration dependence of the initial rate of  $\text{Na}_2\text{SO}_4$ -induced collapse of the pH gradient imposed by a pH jump in liposomes clamped at  $-120$  mV. Inset: Hanes-Woolf plot of the data. Data taken from the experiment shown in Fig. 3.14A.

The results presented so far have shown that measurements of the pH gradient gave no evidence to support the presence of a  $\text{Na}^+/\text{H}^+$  antiport in the tonoplast vesicles. However, Rea et al. (1987b) demonstrated that imposition of a 2 unit jump (between pH 6.0 and pH 8.0) in tonoplast vesicles led to uptake of  $\text{Na}^+$ , measured directly using  $^{22}\text{Na}$ . To see if there were any artefacts associated with this approach, pH jump experiments were carried out in liposomes using  $^{22}\text{Na}$  uptake to measure  $\text{Na}^+$  transport (Fig. 3.16). After the addition of liposomes, labelled  $\text{Na}_2\text{SO}_4$  was added and subsequent uptake of  $^{22}\text{Na}$  was determined. A low concentration of  $\text{Na}^+$  (0.1 mM) was used so as to maintain a high specific activity of  $^{22}\text{Na}$  (Haigh and Phillips 1989). When liposomes loaded at pH 8.0 were diluted into medium at pH 8.0, subsequent addition of labelled  $\text{Na}_2\text{SO}_4$  led to a small amount of  $\text{Na}^+$  uptake. When the liposomes were loaded at pH 6.0 uptake of  $\text{Na}^+$  was much greater and this was

further enhanced by valinomycin. These results show that the pH jump technique is unsuitable for assaying a  $\text{Na}^+/\text{H}^+$  antiporter by measuring  $^{22}\text{Na}$  uptake, as  $^{22}\text{Na}$  uptake was observed in a protein-free membrane system. These results also show directly that valinomycin increases the rate of  $\text{Na}^+$  transport.

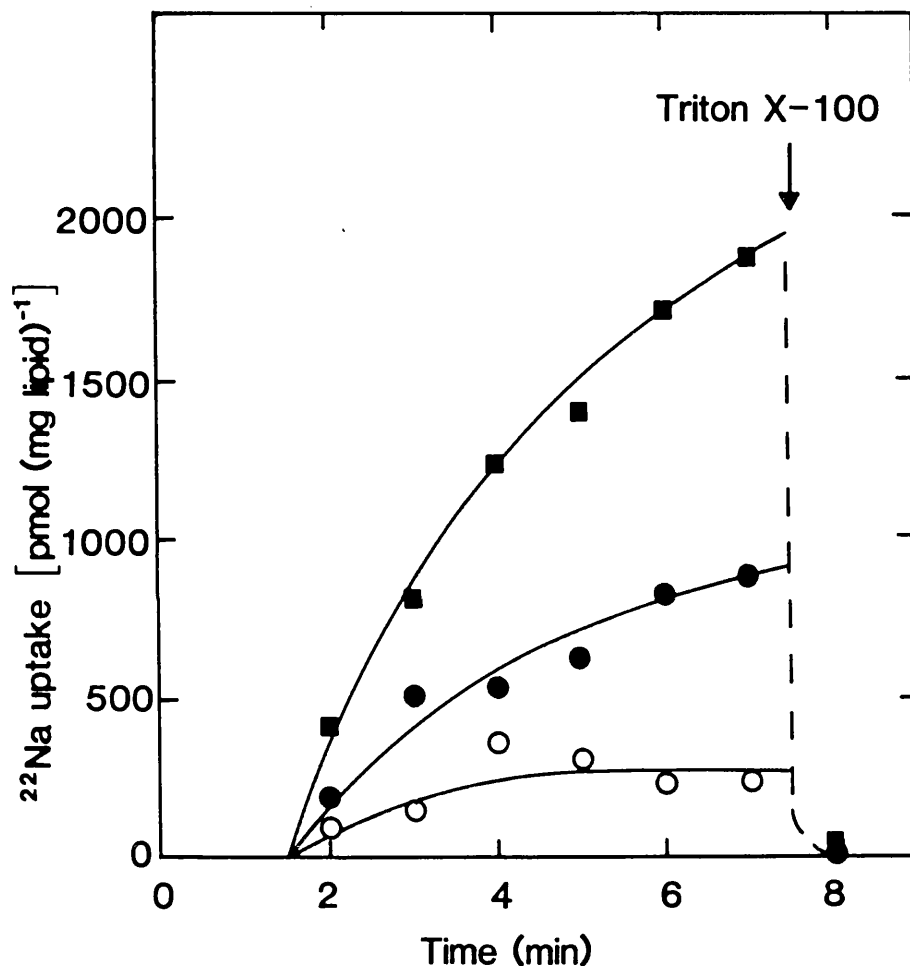


Fig. 3.16. Accumulation of  $^{22}\text{Na}$  by liposomes. Liposomes were loaded with 0.5 M sorbitol, 1 mM EDTA and 5 mM BTP-MES pH 6.0 (■,●) or 0.47 M sorbitol, 0.3 mM EGTA, 10 mM BTP-HEPES pH 8.0 (○) and added to 0.47 M sorbitol, 0.3 mM EGTA, 10 mM BTP-HEPES pH 8.0 at  $t=0$ , with (■) or without (●,○) 5  $\mu\text{M}$  valinomycin. Labelled  $\text{Na}_2\text{SO}_4$  was added at  $t=1.5$  min to give a  $\text{Na}^+$  concentration of 0.1 mM. Aliquots of 100- $\mu\text{l}$  were removed at intervals and filtered and washed as described in 3.2.5. Triton X-100 was added as indicated at a concentration of 0.015% (w/v).



### 3.4 Discussion

The pH jump experiments performed under the conditions used by Blumwald and Poole (1985a), without a voltage clamp, did not provide any evidence for a  $\text{Na}^+/\text{H}^+$  antiport. The controls performed in the absence of a pH gradient showed that the quench on addition of vesicles and the recovery on addition of  $\text{Na}_2\text{SO}_4$  were independent of the pH gradient. These data highlight the need for caution in accepting data obtained using fluorescent probes at face value. The behaviour of a fluorescent probe should always be checked under the exact conditions under which it is to be used. The large changes in fluorescence obtained with acridine orange in these conditions were probably due to binding effects. If acridine orange was binding to the vesicles in the absence of  $\text{Na}_2\text{SO}_4$ , causing a quenching of fluorescence, and displaced from the vesicles by  $\text{Na}_2\text{SO}_4$ , causing a recovery of fluorescence, then all these observations in Figs. 3.1-3 would be accounted for. Garborino and DuPont (1988) also reported similar problems with barley tonoplast vesicles. Acridine orange bound to barley tonoplast vesicles and was displaced by salts, causing a quenching of fluorescence on addition of vesicles in the absence of an imposed pH gradient and a recovery of fluorescence on addition of salts.

Measurement of the pH gradient by [ $^{14}\text{C}$ ]methylamine under the conditions used by Blumwald and Poole (1985a), without a voltage clamp, showed that the pH gradient was very unstable, even in the absence of  $\text{Na}^+$ . The instability of the pH gradient in these conditions is probably due to the presence of Tris buffer, which has been observed to collapse pH gradients in vesicle systems (Sone et al 1977).

With modifications to the media used for pH jumps, conditions were found in which the pH gradient was stable and in which acridine orange fluorescence reported the pH gradient correctly. The results obtained with acridine orange agreed with those obtained using [<sup>14</sup>C]methylamine and controls with acridine orange in the absence of an imposed pH gradient gave only small changes in fluorescence.

The composition of the medium used for pH jump experiments determined whether or not changes in acridine orange fluorescence reported the pH gradient correctly. Binding of the probe only appeared to be a problem under the conditions used by Blumwald and Poole (1985a). Under the modified conditions it seems that another species, probably BTP, was binding to the vesicles in preference to acridine orange, so that the probe was able to report the pH gradient correctly.

Under the modified conditions, in the presence of a K<sup>+</sup>/valinomycin membrane potential clamp, the pH gradient was unstable at 0 mV but stable at -120 mV, as found previously by Rea et al. (1987b). This result shows that the tonoplast vesicles are leaky to H<sup>+</sup>, so that H<sup>+</sup> moves until the electrochemical gradient for H<sup>+</sup> is 0. The electrochemical gradient for H<sup>+</sup> is given by the following equation:

$$\Delta p = \Delta \psi - 60[\text{pH}_i - \text{pH}_o] \quad \text{at } 25^\circ\text{C}$$

where  $\Delta p$  is the electrochemical gradient for H<sup>+</sup>,  $\Delta \psi$  the membrane potential,  $\text{pH}_o$  the pH outside the vesicle and  $\text{pH}_i$  the pH inside the vesicle. At a membrane potential of 0 mV, an electrochemical gradient for H<sup>+</sup> of 0 is reached when there is no pH gradient, so

the pH gradient collapses. At a membrane potential of  $-120$  mV, an electrochemical gradient for  $H^+$  of 0 is reached when there is a 2 unit inside acid pH gradient, so a 2 unit pH gradient is stable. This argument also explains why the pH gradient was stable in the absence of an imposed membrane potential. Leakage of  $H^+$  in the absence of any permeable counter ions will lead to an inside-negative  $H^+$  diffusion potential, which will get more negative until the equilibrium potential is reached, when the pH gradient will be stable. The lack of effect of gramicidin on the pH gradient in the absence of a clamp or when clamped at  $-120$  mV is also consistent with this interpretation. The stability of the pH gradient under these conditions is not dependent on a low conductance to  $H^+$  but on a negative membrane potential. Increasing the already high conductance of the vesicles to  $H^+$  by the addition of gramicidin would not be expected to have any effect on the stability of the pH gradient.

Blumwald and Poole (1985a) used a  $K^+$ /valinomycin voltage clamp at 0 mV for some of their experiments. They did not present any fluorescence traces of pH jumps clamped at 0 mV in this paper but in later work (Blumwald and Poole 1986; Blumwald et al. 1987) presented traces of acridine orange fluorescence from pH jumps clamped at 0 mV. These traces were interpreted as showing that there was a population of vesicles that was leaky to  $H^+$  and could not hold a pH gradient and a population that was sealed to  $H^+$  and could hold a pH gradient. However, the problems observed here with acridine orange in the media used by Blumwald and Poole (1985a) must cast doubt on this interpretation.

The pH jump experiments performed here in the presence of  $K^+$

and valinomycin, intended to clamp the membrane potential at -120 mV, did not provide any evidence for a  $\text{Na}^+/\text{H}^+$  antiport. Although the pH gradient was collapsed by  $\text{Na}^+$  and the initial rate of collapse showed Michaelis-Menten kinetics, these results were also obtained when phosphatidylcholine liposomes were used instead of tonoplast vesicles. Valinomycin directly increased the rate of  $\text{Na}^+/\text{H}^+$  exchange, independently of its role as a  $\text{K}^+$  ionophore. The results with  $^{22}\text{Na}$  in liposomes show that valinomycin increased the rate of  $\text{Na}^+$  transport as well as  $\text{H}^+$  transport.

The pH jump experiments performed in the absence of valinomycin gave no evidence for a  $\text{Na}^+/\text{H}^+$  antiport. A partial collapse of the pH gradient was seen in both tonoplast vesicles and liposomes. The collapse of the pH gradient seen in tonoplast is probably due to the properties of the lipid bilayer, rather than any  $\text{Na}^+$  transport system. Rea et al. (1987b) showed that tonoplast vesicles accumulated  $^{22}\text{Na}$  in response to a pH gradient imposed by a pH jump. However, as this was seen in liposomes (Fig. 3.16), accumulation of  $^{22}\text{Na}$  in response to a pH jump does not necessarily indicate that a  $\text{Na}^+/\text{H}^+$  antiport is present.

In conclusion, the results presented in this chapter show that the technique of using artificially-imposed gradients is not suitable for studying  $\text{Na}^+/\text{H}^+$  exchange at the tonoplast. The mechanisms behind the observations made here are investigated in the next chapter.

COLLAPSE pH GRADIENTS?

4.1 Introduction

The experiments carried out in the previous chapter revealed a number of problems with the use of pH jumps to measure Na<sup>+</sup>/H<sup>+</sup> exchange. In the absence of valinomycin, phosphatidylcholine liposomes showed Na<sup>+</sup>/H<sup>+</sup> exchange properties that might be ascribed to a Na<sup>+</sup>/H<sup>+</sup> antiport in a vesicle system. In the presence of valinomycin, intended to clamp the membrane potential through a K<sup>+</sup> conductance, these effects were not eliminated but were greater. As pH jump experiments have been widely used in studies of Na<sup>+</sup>/H<sup>+</sup> antiports (3.1), it was considered important to investigate the reason for these findings further. These studies were concerned with how a phospholipid bilayer could itself mediate Na<sup>+</sup>/H<sup>+</sup> exchange and how this could be increased by valinomycin, usually considered to be highly specific for K<sup>+</sup> transport (Pressman 1976). A model explaining these observations in terms of electrophoretic Na<sup>+</sup> movements which are increased by non-specific effects of valinomycin is proposed on the basis of the experiments carried out in this and the previous chapter.

## **4.2 Materials and Methods**

**4.2.1 Preparation of tonoplast vesicles and liposomes.** Tonoplast vesicles for pH jump experiments and liposomes were prepared as described in 3.2.1. and 3.2.3, respectively. Tonoplast vesicles for H<sup>+</sup>-pumping assays were isolated as described in 2.2.2. After removal from the step gradients, vesicles were diluted with resuspension medium, pelleted and resuspended in resuspension medium as described in 2.2.2.

**4.2.2 Measurement of pH gradients in tonoplast vesicles and liposomes.** Measurements were made with acridine orange as described in 3.2.2 and 3.2.4. The pH gradient in tonoplast vesicles generated by the ATPase was measured similarly, except that the fluorescent probe quinacrine (Deamer et al. 1972; Lee and Forte 1978; Lee et al. 1982) was used, with excitation and emission wavelengths of 420 and 495 nm respectively.

**4.2.3 Measurement of the membrane potential in liposomes.** Formation and depolarization of a negative-inside membrane potential was measured using using the fluorescent probe safranin O (Waggoner 1979; Woolley et al. 1987). Experiments were carried out under the same conditions used for measurement of acridine orange fluorescence (3.2.2 and 3.2.4), except that the excitation and emission wavelengths were 520 and 585 nm respectively and slit widths were 5 and 10 nm respectively. Safranin O was used routinely at a concentration of 0.1  $\mu$ M.

**4.2.4 Other methods.** Protein was assayed as described in 2.2.4 and ionophores were prepared and used as described in 3.2.7.

### 4.3 Results

4.3.1 Concentration dependence of the effect of valinomycin on  $\text{Na}^+/\text{H}^+$  exchange. Experiments in the previous chapter showed that 5  $\mu\text{M}$  valinomycin more than doubled the initial rate of  $\text{Na}^+$ -induced collapse of the pH gradient imposed by a pH jump in tonoplast vesicles (Fig. 3.12). When the valinomycin concentration dependence of this effect was examined, it was found that valinomycin concentrations above 0.1  $\mu\text{M}$  were required (Fig. 4.1). Below this concentration valinomycin had no effect. This observation suggested that a non-specific effect of valinomycin at these relatively high concentrations could be responsible.

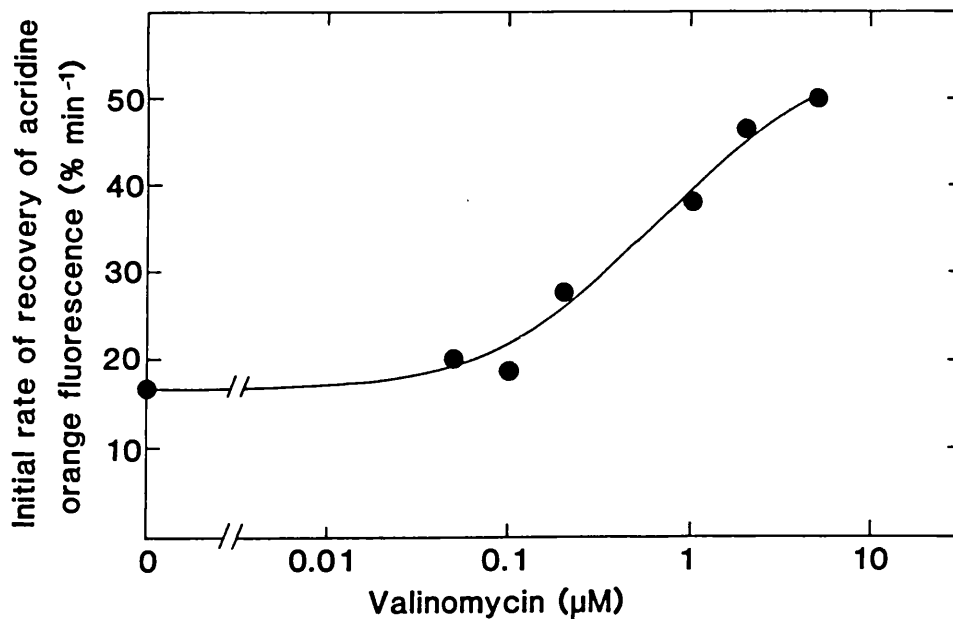
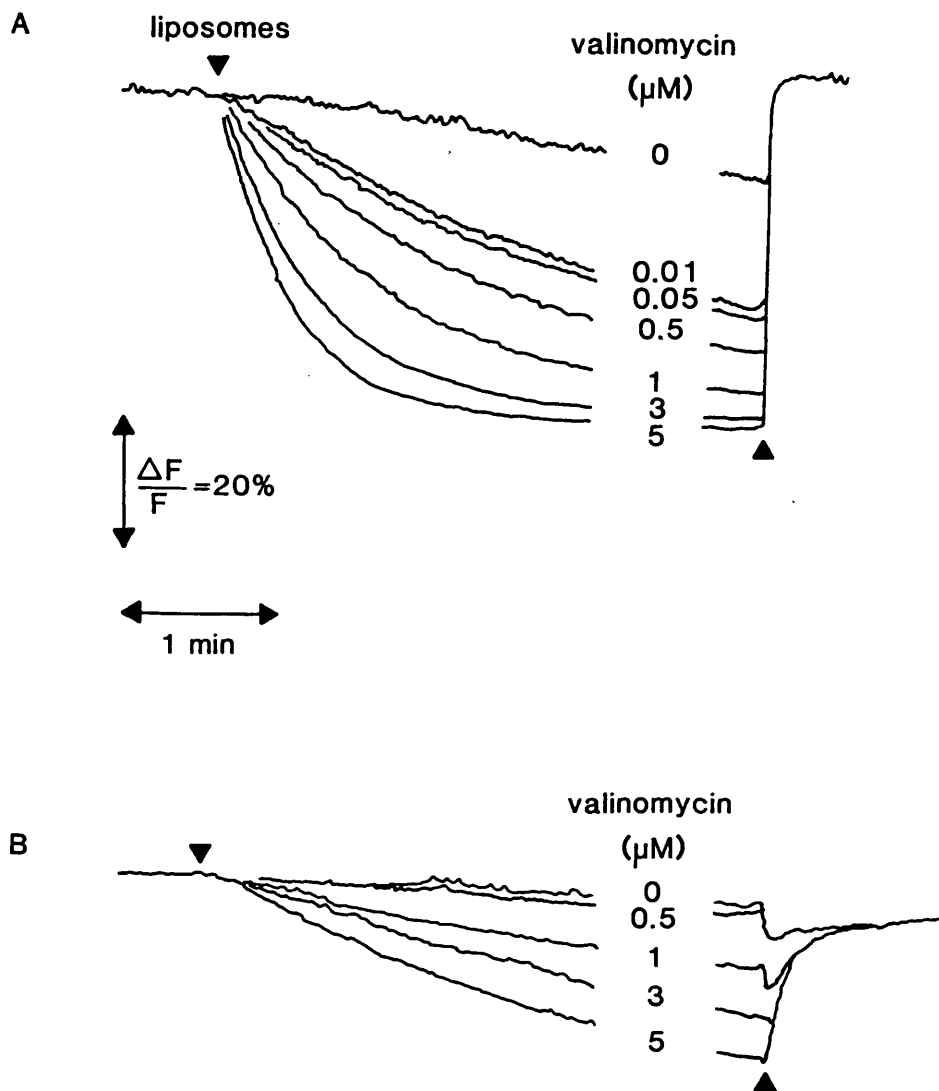


Fig. 4.1. Effect of valinomycin concentration on the collapse of a pH jump-imposed pH gradient by  $\text{Na}_2\text{SO}_4$  in tonoplast vesicles. Vesicles were loaded with 0.5 M sorbitol, 20 mM  $\text{K}_2\text{SO}_4$ , 1 mM EDTA, 2 mM DTT, 5 mM BTP-MES pH 5.5 and diluted 100-fold into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu\text{M}$  acridine orange, 10 mM BTP-HEPES pH 7.5, containing different concentrations of valinomycin or a control amount of ethanol. After addition of vesicles,  $\text{Na}_2\text{SO}_4$  was added 1.5 min later to give 95 mM  $\text{Na}^+$  and the initial rate of recovery of acridine orange fluorescence was determined.

The possibility that the effect of valinomycin was due to a non-specific effect at relatively high concentrations was tested further in experiments in liposomes. Garcia et al. (1984) found that when liposomes were loaded with  $\text{Na}^+$  and diluted into  $\text{Na}^+$ -free medium in the presence of valinomycin at  $\mu\text{M}$  concentrations, a  $\text{Na}^+$  diffusion potential was established which led to pH gradient formation. Experiments were therefore performed with liposomes to see if this effect could account for the effects of valinomycin observed in Chapter 3.

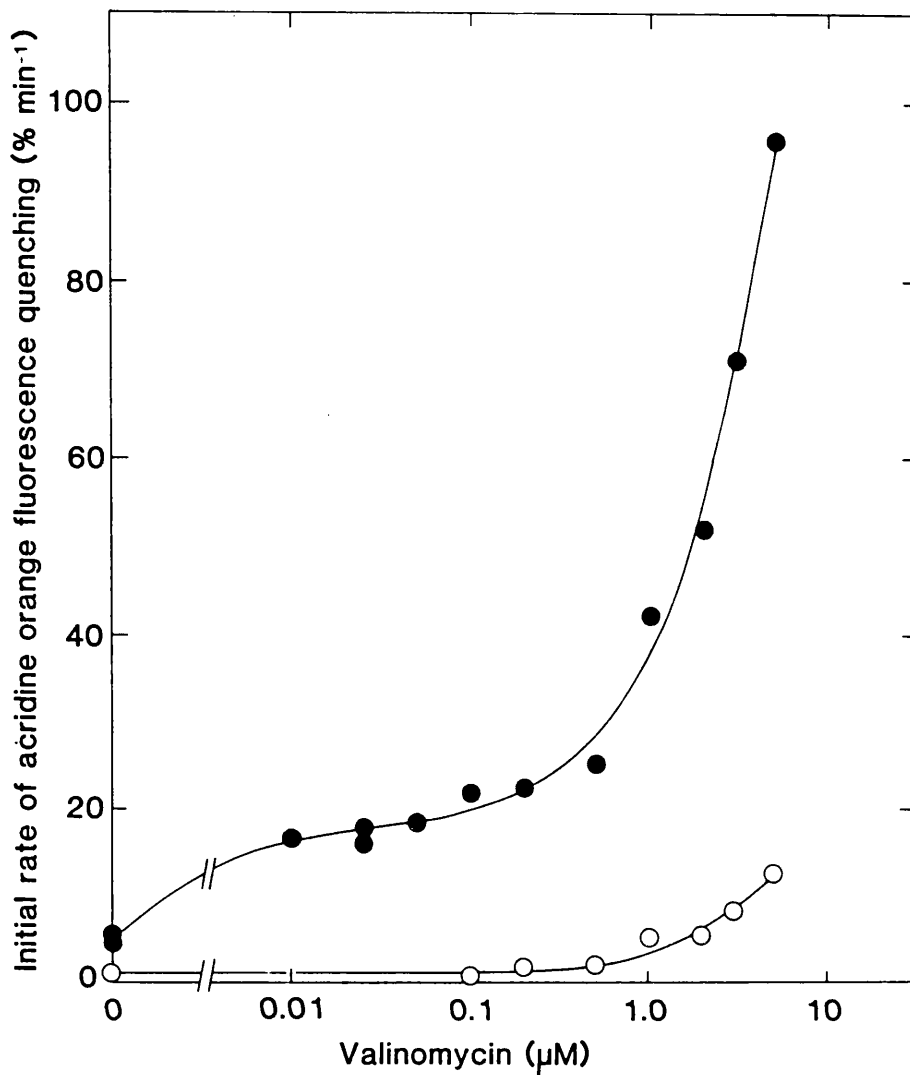
Liposomes were loaded with 50 mM  $\text{K}_2\text{SO}_4$  and diluted 100-fold into medium at the same pH containing valinomycin to impose a  $\text{K}^+$  diffusion potential of -120 mV. As expected (Garcia et al. 1984), the addition of liposomes to medium containing valinomycin led to pH gradient formation as the  $\text{K}^+$  diffusion potential (inside-negative) provided a driving force for  $\text{H}^+$  uptake (Fig. 4.2A). Concentrations of valinomycin as low as 10 nM stimulated pH gradient formation compared with a control without valinomycin. Increasing the valinomycin concentration to 0.1  $\mu\text{M}$  had little effect on the initial rate of pH gradient formation (Fig. 4.3) but at higher concentrations of valinomycin both the initial rate and extent of pH gradient formation increased rapidly with valinomycin concentration. Liposomes were also loaded with 50 mM  $\text{Na}_2\text{SO}_4$  and diluted 100-fold into medium containing valinomycin. In the absence of valinomycin no pH gradient was formed (Fig. 4.2B). Concentrations of valinomycin below 0.5  $\mu\text{M}$  had no effect on pH gradient formation. Higher concentrations led to pH gradient formation and the initial rate of pH gradient formation increased with increasing valinomycin concentration (Fig. 4.3). The effect of valinomycin was therefore specific to  $\text{K}^+$  at concentrations below 0.5  $\mu\text{M}$ .





**Fig. 4.2A,B.** Formation of pH gradients in liposomes by outwardly-directed K<sup>+</sup> or Na<sup>+</sup> gradients, measured by the fluorescence quenching of acridine orange. Liposomes loaded with 0.4 M sorbitol, 1 mM EDTA, 5 mM BTP-MES pH 7.5 and either 50 mM K<sub>2</sub>SO<sub>4</sub> (A) or 50 mM Na<sub>2</sub>SO<sub>4</sub> (B) were diluted 100-fold into 0.47 M sorbitol, 0.3 mM EGTA, 5  $\mu\text{M}$  acridine orange, 10 mM BTP-HEPES pH 7.5 containing valinomycin at the indicated concentration. As indicated by ( $\blacktriangle$ ), the pH gradient was collapsed by the addition of 0.015% Triton X-100 in (A) or 50 mM Na<sub>2</sub>SO<sub>4</sub> in (B).

These results suggested that the effects of valinomycin on Na<sup>+</sup> transport described in the last chapter were due to non-specific effects of valinomycin at the concentration used. It appeared that high valinomycin concentrations were increasing the Na<sup>+</sup> permeability of the bilayer. In the presence of valinomycin, a



**Fig. 4.3.** Initial rate of acridine orange fluorescence quenching in response to a 100-fold outwardly-directed K<sup>+</sup> gradient (●) or Na<sup>+</sup> gradient (○) in liposomes as a function of the valinomycin concentration. Data taken from Fig. 4.2.

diffusion potential would be generated that would drive H<sup>+</sup> influx in the same way as for K<sup>+</sup> (Garcia et al. 1984).

The above experiments suggested an explanation for how Na<sup>+</sup> and valinomycin were able to collapse pH gradients in pH jump experiments. The stability of pH gradients in both tonoplast and liposomes depended upon the presence of a negative membrane potential (Chapter 3; Figs. 3.5, 3.6, 3.13). If conductive movements of Na<sup>+</sup> in the presence of valinomycin were able to depolarise this

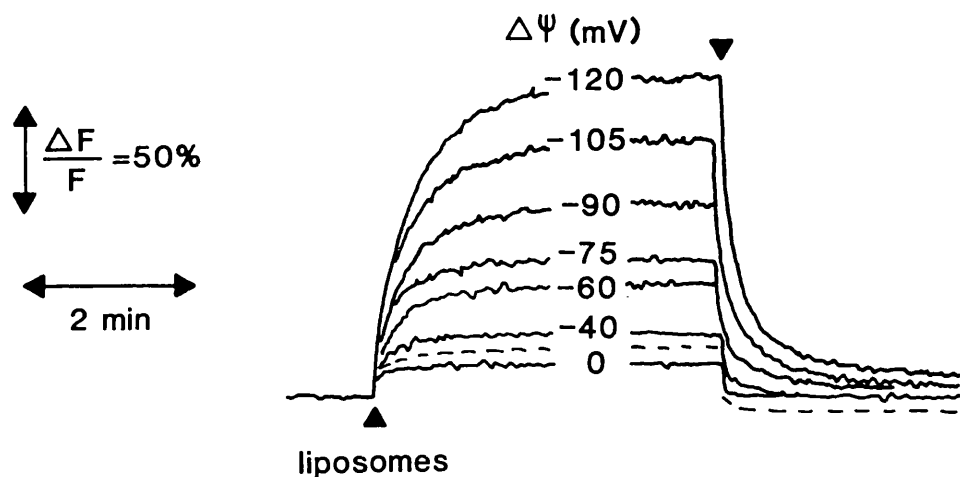
potential, then the pH gradient would collapse. This hypothesis was tested by making measurements of the membrane potential in pH jump experiments using the fluorescent probe safranin O.

**4.3.2 The use of safranin O to measure negative membrane potentials.** Safranin O has been used to monitor inside-negative membrane potentials in a variety of biological systems and in liposomes (Waggoner, 1979). At probe concentrations above 1  $\mu\text{M}$  the fluorescence of safranin O is quenched in the presence of an inside-negative membrane potential. However, at a concentration of 1  $\mu\text{M}$  the fluorescence of safranin O is enhanced in the presence of an inside-negative membrane potential (Woolley et al. 1987). Experiments were therefore performed to optimise the conditions for using safranin O to make measurements of membrane potentials.

Liposomes were loaded with 50 mM  $\text{K}_2\text{SO}_4$  and diluted 100-fold into medium at the same pH containing 0.25  $\mu\text{M}$  valinomycin to impose a  $\text{K}^+$  diffusion potential of -120 mV. The fluorescence of 5  $\mu\text{M}$  safranin O was quenched by about 20% on addition of liposomes, indicating the formation of a membrane potential (not shown). Using safranin O concentrations higher or lower than 5  $\mu\text{M}$  gave smaller quenches on addition of vesicles (not shown). When safranin O was used at a concentration of 2  $\mu\text{M}$ , there was no net change in fluorescence after addition of liposomes. When safranin O was used at a concentration of 1  $\mu\text{M}$ , addition of liposomes caused the fluorescence to increase by about 50% over the value in the absence of liposomes, as found by Woolley et al. (1987) (not shown). Using probe concentrations below 1  $\mu\text{M}$  gave a larger enhancement of fluorescence, with a concentration of 0.1  $\mu\text{M}$  giving a fluorescence enhancement of 150% over the original value (Fig. 4.4). Using

safranin O concentrations lower than 0.1  $\mu\text{M}$  gave only a slightly larger enhancement of fluorescence and gave noticeably more noisy results so 0.1  $\mu\text{M}$  safranin O was used routinely.

To check that the enhancement of safranin O fluorescence was responding to the membrane potential, the response of the probe to a range of  $\text{K}^+$  diffusion potentials was examined. Diffusion potentials of differing magnitude were imposed by varying the  $\text{K}^+$  concentration in the medium to which the liposomes were added and the assumed membrane potential was calculated from the Nernst equation. The enhancement of safranin O fluorescence was found to vary in a potential-dependent manner (Fig. 4.4). On addition of  $\text{K}_2\text{SO}_4$  to depolarise the membrane potential, the fluorescence signal returned to its starting value. On addition of liposomes



**Fig. 4.4.** Enhancement of safranin O fluorescence in the presence of  $\text{K}^+$ /valinomycin diffusion potentials and return to starting signal on depolarisation of the membrane potential with  $\text{K}_2\text{SO}_4$ . Liposomes were loaded with 0.4 M sorbitol, 1 mM EDTA, 50 mM  $\text{K}_2\text{SO}_4$  and 5 mM BTP-MES pH 7.5 and diluted 100-fold into a medium containing 0.47 M sorbitol, 0.3 mM EGTA, 0.1  $\mu\text{M}$  valinomycin, 0.1  $\mu\text{M}$  safranin O, 10 mM BTP-HEPES pH 7.5 and  $\text{K}_2\text{SO}_4$  at a concentration calculated by the Nernst equation to give the indicated membrane potential. As indicated, an aliquot of  $\text{K}_2\text{SO}_4$  at the appropriate concentration was added to depolarise the membrane potential. The addition of liposomes prepared without  $\text{K}_2\text{SO}_4$  and added to medium containing 0.5 mM  $\text{K}_2\text{SO}_4$  is indicated by (---).

containing no  $K^+$  to medium containing 1 mM  $K^+$  there was only a small enhancement of fluorescence, showing that the probe was responding to the membrane potential and not simply to the external  $K^+$  concentration.

The steady-state enhancement of fluorescence obtained from the results shown in Fig. 4.4 was plotted against the membrane potential (Fig. 4.5). Over the range of membrane potentials examined the response of the probe was curvilinear, showing greater sensitivity as the magnitude of the potential was increased. When the log of fluorescence enhancement was plotted against membrane potential a straight line was obtained (Fig. 4.5 inset).

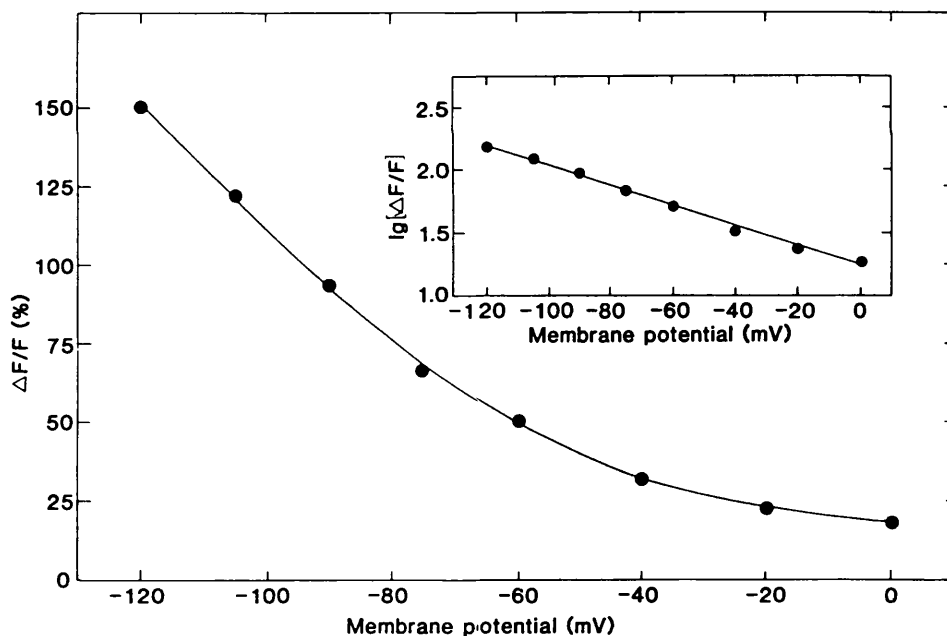
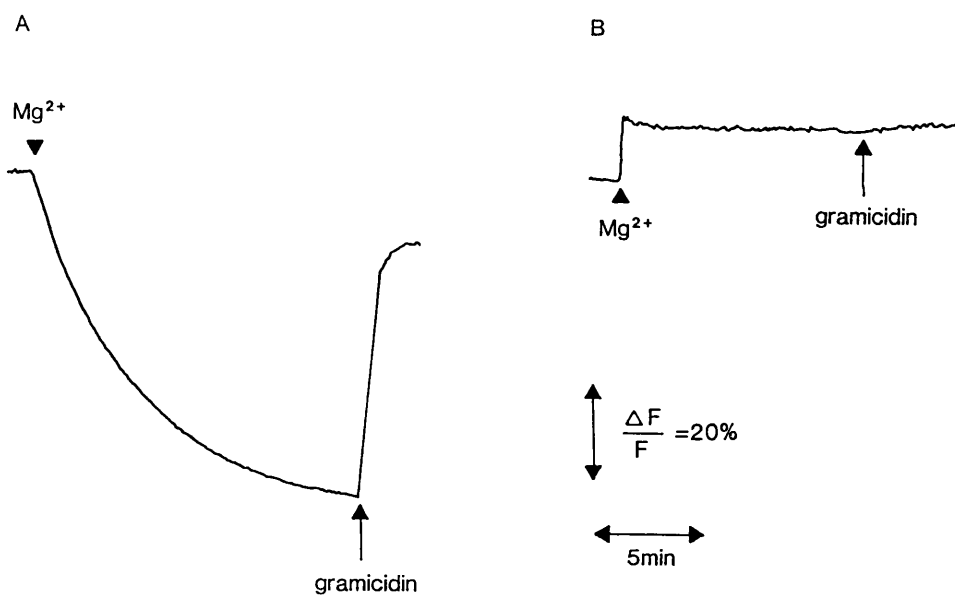


Fig. 4.5. Enhancement of safranin O fluorescence against calculated membrane potential. Data taken from results shown in Fig. 4.4. Inset: log plot of the data.

As the imposition of an inside-negative  $K^+$  diffusion potential drives pH gradient formation (Fig. 4.2A), the possibility that

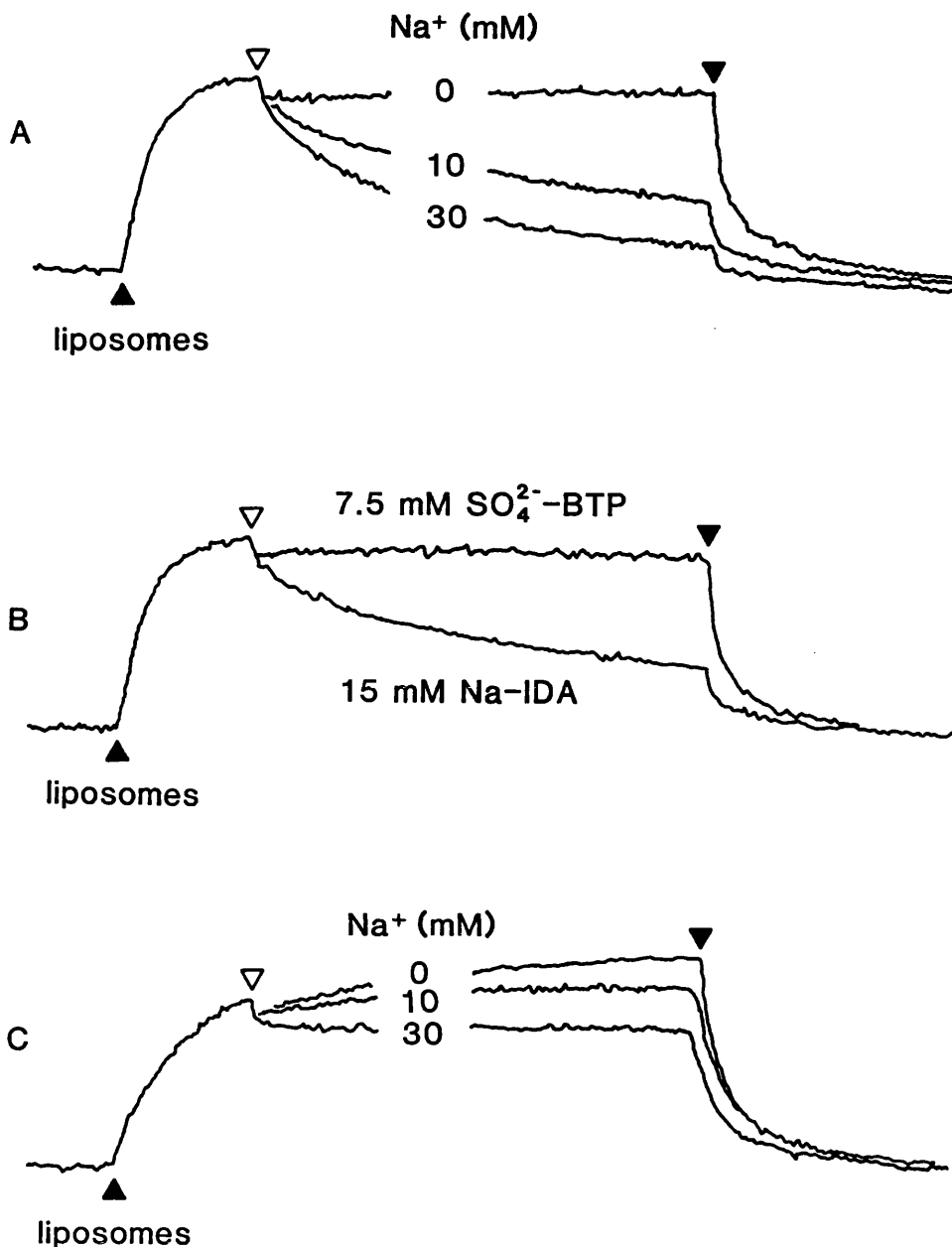
safranin O could be responding to the pH gradient rather than to the membrane potential was investigated. This was done by generating a pH gradient in the absence of a negative membrane potential in tonoplast vesicles using the H<sup>+</sup>-ATPase. The fluorescence quenching of quinacrine showed that an inside-acid pH gradient was generated and the fluorescence was recovered on addition of gramicidin (Fig. 4.6A). When 0.1 μM safranin O was used, there was only a 15% enhancement of fluorescence on addition of MgSO<sub>4</sub> and this was not recovered by gramicidin, showing that safranin O was not responding to the pH gradient (Fig. 4.6B). These results show that the effects previously observed can be attributed to the membrane potential.



**Fig. 4.6A,B.** ATP-dependent H<sup>+</sup>-pumping in tonoplast vesicles. Demonstration of inside-acid pH gradient formation with 5 μM quinacrine, showing no response with 0.1 μM safranin O. Vesicles in resuspension medium were added to a medium containing 0.25 M glycerol, 50 mM KCl, 1.5 mM ATP, 5 mM BTP-MES pH 8.0 containing either 5 μM quinacrine (A) or 0.1 μM safranin O (B). The H<sup>+</sup>-ATPase was activated by addition of 1.5 mM MgSO<sub>4</sub> as indicated. Gramicidin was added as indicated at a concentration of 2.5 μg ml<sup>-1</sup>.

Safranin O was used to monitor the membrane potential in pH jump experiments. When a pH jump was imposed on liposomes in the presence of a 100-fold outwardly-directed  $K^+$  gradient and 5  $\mu M$  valinomycin, intended to clamp the membrane potential at -120 mV, safranin O fluorescence was enhanced by 90%, indicating the formation of a negative membrane potential (Fig. 4.7A). Addition of  $Na_2SO_4$  caused the membrane potential to depolarise, and both the rate and extent of depolarisation were greater with 30 mM  $Na^+$  than with 10 mM  $Na^+$ . This shows directly that the  $K^+$ /valinomycin clamp was not maintaining a negative membrane potential in the presence of  $Na^+$ . Figure 4.7B shows that  $Na^+$  rather than sulphate was responsible for this effect: the membrane potential was stable in the presence of sulphate-BTP but not in the presence of Na-IDA.

In the absence of valinomycin, there was an enhancement of safranin O fluorescence on imposition of a pH gradient (Fig. 4.7C). This could be due to an  $H^+$  diffusion potential or a  $K^+$  diffusion potential, or both. This negative potential developed more slowly than the potential imposed by  $K^+$  and valinomycin (compare with Fig. 4.7A). The fluorescence signal was stable when 30 mM  $Na^+$  was added but continued increasing to reach a value of 100% in the absence of  $Na^+$ , and increased to a lesser extent with 10 mM  $Na^+$ . This shows that the effect of  $Na^+$  on the membrane potential is much smaller in the absence of valinomycin. In all the recordings shown in Fig. 4.7 the fluorescence returned to approximately its starting value on addition of 25 mM  $K_2SO_4$  and 0.5  $\mu g\ ml^{-1}$  gramicidin to collapse the pH gradient and depolarise the membrane potential. Triton X-100 was not used for this purpose as it interferes with safranin O fluorescence (not shown). As a control, liposomes loaded at pH 7.5 without  $K_2SO_4$  were added to medium



**Fig. 4.7A,B,C.** Measurement of membrane potential in pH jumps in liposomes with safranin O fluorescence. A 2 unit pH jump was imposed by diluting liposomes loaded with 0.5 M sorbitol, 1 mM EDTA, 20 mM K<sub>2</sub>SO<sub>4</sub>, 5 mM BTP-MES pH 5.5 100-fold into a medium containing 0.47 M sorbitol, 0.3 mM EGTA, 0.1 μM safranin O, 10 mM BTP-HEPES pH 7.5 and either with (A,B) or without (C) 5 μM valinomycin. In (A) and (C) Na<sub>2</sub>SO<sub>4</sub> or sorbitol was added as indicated by (▽) to give the indicated Na<sup>+</sup> concentration. In (B) the indicated salt was added at (▽). The membrane potential was collapsed at the indicated time (▼) by addition of 25 mM K<sub>2</sub>SO<sub>4</sub> and 2.5 μg ml<sup>-1</sup> gramicidin.



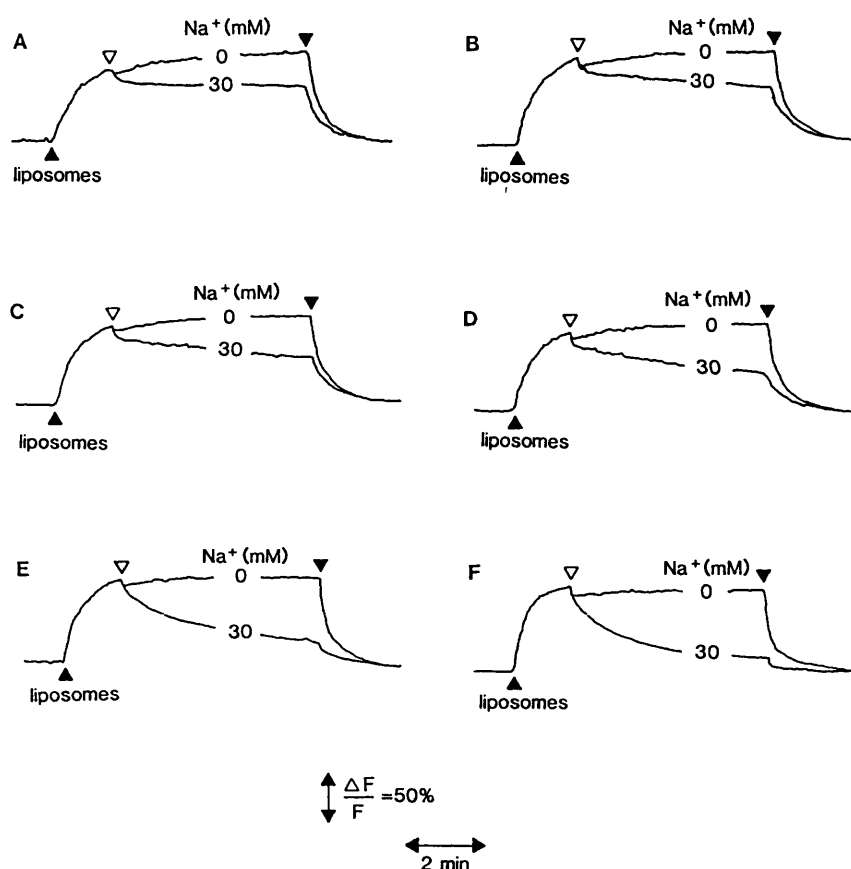
containing safranin O and 0.2 mM  $K_2SO_4$ . The enhancement of fluorescence on addition of liposomes was only 18% (not shown).

Similar responses of safranin O to  $Na^+$  and valinomycin were observed in the absence of  $K_2SO_4$  in pH jump experiments (not shown) although addition of liposomes gave a  $\Delta F/F$  of 116% in the absence of  $Na^+$  and valinomycin, compared with a  $\Delta F/F$  of 100% in the presence of a 100-fold outwardly-directed  $K^+$  gradient. However, when liposomes prepared without  $K^+$  at pH 7.5 were added to a  $K^+$ -free medium at pH 7.5, the  $\Delta F/F$  was 37% (compared with 18% when the medium contained 0.4 mM  $K^+$ ). This suggests that the larger values of  $\Delta F/F$  obtained in pH jumps in the absence of  $K^+$  (compared to pH jumps in the presence of a 100-fold outwardly-directed  $K^+$  gradient) were not due to an increased membrane potential.

Attempts to make measurements with safranin O in pH jumps in tonoplast vesicles were not successful. Large changes in fluorescence were observed on the addition of vesicles to media containing safranin O, even in the absence of any imposed pH gradient or membrane potential. These effects were probably due to binding of the probe to the vesicles.

As a previous experiment (Fig. 4.2) showed that valinomycin appeared to be specific for  $K^+$  at low valinomycin concentrations, experiments were done to test whether a  $K^+$ /valinomycin clamp would hold the membrane potential in the presence of  $Na^+$  when valinomycin was used at low concentrations. If the membrane potential was being held by the clamp in the presence of  $Na^+$ , then the potential should be the same with  $Na^+$  as without  $Na^+$ .

Fig. 4.8 shows that this did not happen even when low concentrations of valinomycin were used. The presence of low concentrations of valinomycin did not increase the stability of the membrane potential in the presence of 30 mM Na<sup>+</sup>. At valinomycin concentrations of 0.1 μM or above there was an increased depolarisation of the membrane potential. These experiments suggested that it is not possible to clamp the membrane potential at -120 mV in the presence of Na<sup>+</sup>, even when using valinomycin at a concentration where there are no non-specific effects.



**Fig. 4.8A,B,C,D,E,F.** An attempt to clamp the membrane potential at -120 mV in the presence of Na<sub>2</sub>SO<sub>4</sub> by using low concentrations of valinomycin. Liposomes were loaded with 0.55 M sorbitol, 20 mM K<sub>2</sub>SO<sub>4</sub>, 1 mM EDTA and 5 mM BTP-MES pH 5.5 and added to a medium containing 0.47 M sorbitol, 0.3 mM EGTA, 0.1 μM safranin O, 10 mM BTP-HEPES pH 7.5 to give a final concentration of 0.2 mM K<sub>2</sub>SO<sub>4</sub>. Valinomycin was present as follows: (A) 0 μM ethanol control; (B) 0.01 μM; (C) 0.05 μM; (D) 0.1 μM; (E) 0.5 μM; (F) 5 μM. Sodium sulphate or sorbitol was added at (▽) to give the indicated Na<sup>+</sup> concentration. The membrane potential was collapsed at the indicated time (▼) by addition of 20 mM K<sub>2</sub>SO<sub>4</sub> and 2.5 μg ml<sup>-1</sup> gramicidin.

#### 4.4 Discussion

The lack of specificity for  $K^+$  over  $Na^+$  in the responses to high valinomycin concentrations was surprising at first, as valinomycin is usually considered to be very specific for  $K^+$  over  $Na^+$ ; Pressman (1976) quotes a selectivity of  $10^5$ . However values as high as this do not come from transport studies but from studies of the partitioning of cation/valinomycin complexes between different solvents. Haynes and Pressman (1974) found values of K/Na selectivity by this approach that varied between 59,000 and 42 depending on the solvent and the anion used. Mueller and Rudin (1967) measured the ability of valinomycin to transport ions across a lipid bilayer. At a valinomycin concentration of 1  $\mu M$ , they measured a K/Na selectivity of 400.

At valinomycin concentrations in the  $\mu M$  range there is evidence for non-specific effects of valinomycin. LaBelle (1984) observed that 7  $\mu M$  valinomycin stimulated  $Na^+$  transport into rabbit kidney medulla microsomes in the absence of  $K^+$ . LaBelle also reported that 18  $\mu M$  valinomycin transported  $Na^+$  into phospholipid liposomes. Woolley et al. (1987) reported that valinomycin concentrations above 3  $\mu M$  caused non-specific ion leakage. Garcia et al. (1984) showed that valinomycin concentrations above 1  $\mu M$  allowed an outwardly-directed  $Na^+$  gradient to drive pH gradient formation in liposomes but that pH gradient formation was more rapid with  $K^+$  loaded liposomes. The results of the experiments shown in Fig. 4.2 are consistent with the findings of Garcia et al. (1984). Valinomycin had little effect on  $Na^+/H^+$  exchange in liposomes below 0.5  $\mu M$ , whereas valinomycin clearly stimulated  $K^+/H^+$  exchange at a concentration of 10 nM. This suggests that the effects

of valinomycin seen here and in Chapter 3 on  $\text{Na}^+/\text{H}^+$  exchange were due to non-specific effects at high concentrations. These non-specific effects may be accounted for by the valinomycin causing a breakdown of membrane integrity. Valinomycin molecules have a hydrophobic surface so they partition from the aqueous phase into the lipid bilayer of a biological membrane or liposome. It is suggested that low concentrations of valinomycin transport  $\text{K}^+$  across the membrane with a high specificity but that high concentrations of valinomycin cause a breakdown of the membrane bilayer. This explanation is supported by some approximate calculations. The experiments with liposomes reported in this chapter used 100  $\mu\text{g}$  lipid per assay in a 2 ml volume. When valinomycin was present at 5  $\mu\text{M}$  each assay would contain 11  $\mu\text{g}$  valinomycin. If it is assumed that 90% of the valinomycin in the system partitions into the bilayer, then the bilayer will contain 10% valinomycin. Such a high proportion of valinomycin would increase the leakiness of the membrane by disrupting the bilayer and therefore increase its  $\text{Na}^+$  permeability.

The experiments with safranin O clearly showed that it was not possible to clamp the membrane potential at -120 mV in the presence of  $\text{Na}^+$ . Instead of clamping the membrane potential, high concentrations of valinomycin actually increased electrophoretic movement of  $\text{Na}^+$  in liposomes. In the absence of valinomycin, the depolarisation of the membrane potential on addition of  $\text{Na}^+$  was much smaller. The results with safranin O also confirmed that a negative  $\text{H}^+$  diffusion potential was formed on imposing a pH gradient in liposomes in the absence of  $\text{K}^+$ /valinomycin. The presence of this  $\text{H}^+$  diffusion potential was deduced in Chapter 3 from the responses of the pH gradient to an imposed membrane

potential.

The measurements with safranin O, together with the measurements of the pH gradient and  $^{22}\text{Na}$  transport presented in Chapter 3, suggest a model to explain the effects of  $\text{Na}^+$  and valinomycin on the stability of pH gradients in tonoplast vesicles and liposomes. The stability of pH gradients in both tonoplast vesicles and liposomes depends upon a negative membrane potential as the membranes are leaky to  $\text{H}^+$  (Chapter 3: Figs. 3.5, 3.6, 3.13). In the presence of valinomycin,  $\text{Na}^+$  enters the vesicle or liposome, driven by its concentration gradient and by the negative membrane potential. This causes a depolarisation of the membrane potential to a less negative value, so the pH gradient collapses. In the absence of valinomycin, the  $\text{Na}^+$  permeability of the membrane is much lower so there is a smaller depolarisation of the membrane potential and therefore a smaller collapse of the pH gradient.

It is concluded that the above model can explain all the observations on  $\text{Na}^+/\text{H}^+$  exchange made in this and the previous chapter. No evidence has been found for a  $\text{Na}^+/\text{H}^+$  antiport in tonoplast vesicles. It appears that artificially-imposed pH gradients do not provide a suitable assay for  $\text{Na}^+/\text{H}^+$  exchange. In the next chapter experiments are described using a different approach to see whether there is a  $\text{Na}^+/\text{H}^+$  antiport at the tonoplast.

## **CHAPTER 5: STUDYING Na<sup>+</sup> TRANSPORT IN RESPONSE TO pH GRADIENTS**

### **GENERATED BY THE H<sup>+</sup>-ATPase**

#### **5.1 Introduction**

The experiments reported in the previous two chapters showed that pH jump experiments were unsuitable for studying Na<sup>+</sup>/H<sup>+</sup> exchange in tonoplast vesicles. The experiments described in this chapter were intended to overcome these problems by using pH gradients generated by the H<sup>+</sup>-ATPase at the tonoplast, measuring the effects of Na<sup>+</sup> on the pH gradient and measuring Na<sup>+</sup> transport directly by using <sup>22</sup>Na.

The approach of using the ATPase to generate a pH gradient, stopping the ATPase by removal of substrate, then looking at the effect of Na<sup>+</sup> on the collapse of the pH gradient, has been used to study Na<sup>+</sup>/H<sup>+</sup> exchange in sugar beet vacuoles (Blumwald et al. 1987) and barley tonoplast vesicles (Garborino and DuPont 1988). Garborino and DuPont (1988) stopped the ATPase in their experiments by adding EDTA to chelate Mg<sup>2+</sup>, thus depleting the substrate, MgATP. They used K<sup>+</sup> and valinomycin to resolve electrically-driven Na<sup>+</sup> and H<sup>+</sup> fluxes from antiport-driven fluxes. They also performed experiments in the presence and absence of Na salts without stopping the ATPase and found that the extent and rate of pH gradient formation was reduced in the presence of NaCl compared with either KCl or choline chloride.

As well as the above approaches it was felt very important to make direct measurements of Na<sup>+</sup> transport in response to pH gradients generated by the ATPase. Generating a pH gradient by the ATPase

gives a stable pH gradient without a negative membrane potential that would provide a driving force for Na<sup>+</sup> uptake through conductive pathways. Any pH gradient-dependent Na<sup>+</sup> uptake seen under these conditions must be due to a Na<sup>+</sup>/H<sup>+</sup> antiport.

## 5.2 Materials and Methods

**5.2.1 Preparation of tonoplast vesicles.** Tonoplast vesicles were isolated from red beet storage roots as described in 2.2.2. After removal from the sucrose step gradients, vesicles were diluted with resuspension medium, pelleted and resuspended in resuspension medium as described in 2.2.2. For the experiment shown in Fig. 5.1 vesicles were loaded with 20 mM  $K_2SO_4$  by including this in the resuspension medium and sucrose gradient solutions. For the experiment shown in Fig. 5.8 the tonoplast vesicles were prepared from beets taken from plants that had been watered with 25 mM NaCl.

**5.2.2 Measurement of pH gradient formation.** The formation of inside-acid pH gradients generated by the ATPase was measured using acridine orange fluorescence or [ $^{14}C$ ]methylamine accumulation as described in 3.2.2. Uptake of [ $^{14}C$ ]methylamine that was dependent on the pH gradient was determined by using a control treatment without ATP. The fluorescent probe quinacrine was also used to monitor pH gradients as described in 4.2.2. The assay conditions for monitoring pH gradient formation are indicated with the results.

**5.2.3 Measurement of membrane potential formation.** The formation of an inside-positive membrane potential generated by the ATPase was monitored by the fluorescence of Oxonol V (Bashford and Smith 1979; Waggoner 1979). Assay conditions were the same as for measurement of the pH gradient, except that excitation and emission wavelengths were 580 and 650 nm respectively and slit widths were 5 and 10 nm respectively. Oxonol V was used at a concentration of 1  $\mu M$ . Oxonol V is very sparingly soluble in distilled water so the stock solution was made up in 50 mM HEPES-BTP pH 9.2.



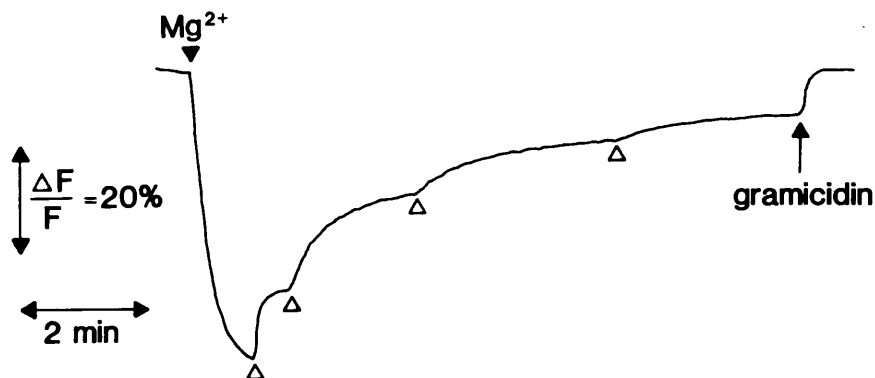
**5.2.4 Measurement of  $^{22}\text{Na}$  uptake by tonoplast vesicles.** Uptake of  $^{22}\text{Na}$  by tonoplast vesicles was measured using a filtration procedure. The assay conditions are indicated with the results but in all experiments 0.1 mM NaCl ( $5 \mu\text{Ci ml}^{-1}$ ) was used. The low  $\text{Na}^+$  concentration was used to maintain a high specific activity of the label. At intervals aliquots were removed from the reaction mixture and filtered and washed as described in 3.2.5. For some experiments glass fibre filters (Whatman GF/F 0.7  $\mu\text{M}$  pore size) were used instead of cellulose nitrate membrane filters. The glass fibre filters were pre-soaked in wash medium for a few hours before use. In all cases the filters were allowed to dry and counted as described in 3.2.5.

**5.2.5 Other methods.** Protein was assayed as described in 2.2.4. and ionophores were prepared and used as described in 3.2.7.

### 5.3 Results

#### 5.3.1 Effects of Na<sup>+</sup> on pH gradients generated by the H<sup>+</sup>-ATPase.

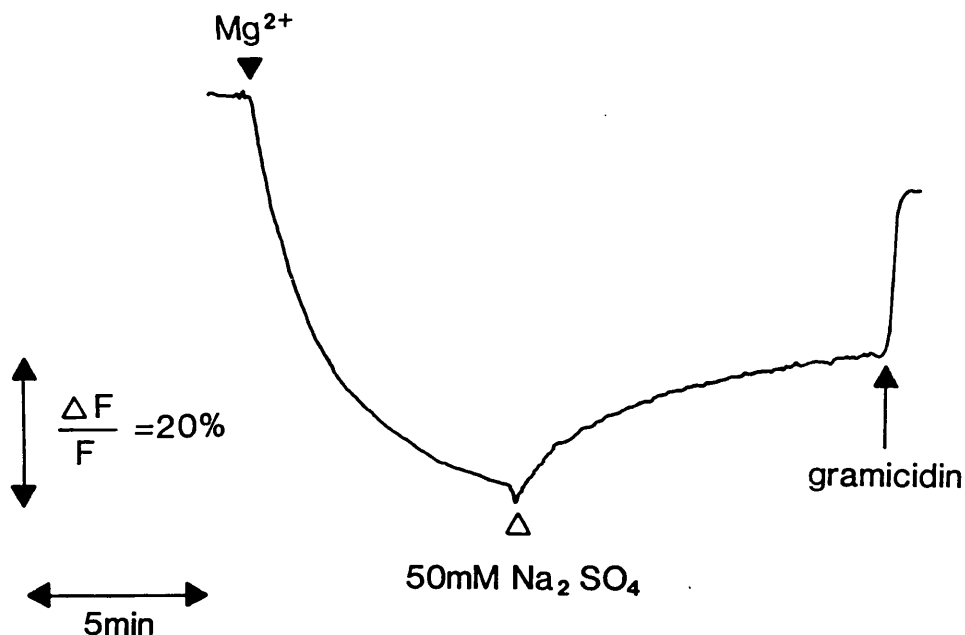
Preliminary experiments showed that EDTA could not be used to stop the ATPase when quinacrine was used as the pH gradient probe as EDTA quenched quinacrine fluorescence (not shown). Acridine orange was therefore used as the pH gradient probe. On addition of MgSO<sub>4</sub> to the reaction mixture, quenching of acridine orange fluorescence indicated the formation of an inside-acid pH gradient (Fig. 5.1). Addition of 2 mM EDTA led to a partial collapse of the pH gradient, but when the EDTA concentration was successively increased by 2 mM, each further increase in concentration led to a further collapse of the gradient. It appeared that low concentrations of EDTA were leaving some of the Mg<sup>2+</sup> unchelated so the ATPase was still active. This raised the possibility that some ATPase activity might



**Fig. 5.1.** ATP-dependent pH gradient formation in tonoplast vesicles and its dissipation by EDTA, measured with acridine orange fluorescence quenching. The reaction mixture consisted of tonoplast vesicles in 0.25 M glycerol, 40 mM KCl, 0.3 mM EGTA, 5  $\mu$ M valinomycin, 0.75 mM ATP-BTP, 5  $\mu$ M acridine orange, 5 mM BTP-MES pH 7.5. The reaction was started as indicated by the addition of 1.5 mM MgSO<sub>4</sub>. At the indicated time ( $\Delta$ ), aliquots of EDTA-BTP pH 7.5 were added to the reaction mixture, each addition increasing the EDTA concentration by 2 mM, to give a final EDTA concentration of 8 mM. At the indicated time 2.5  $\mu$ g ml<sup>-1</sup> gramicidin was added to collapse any remaining pH gradient.

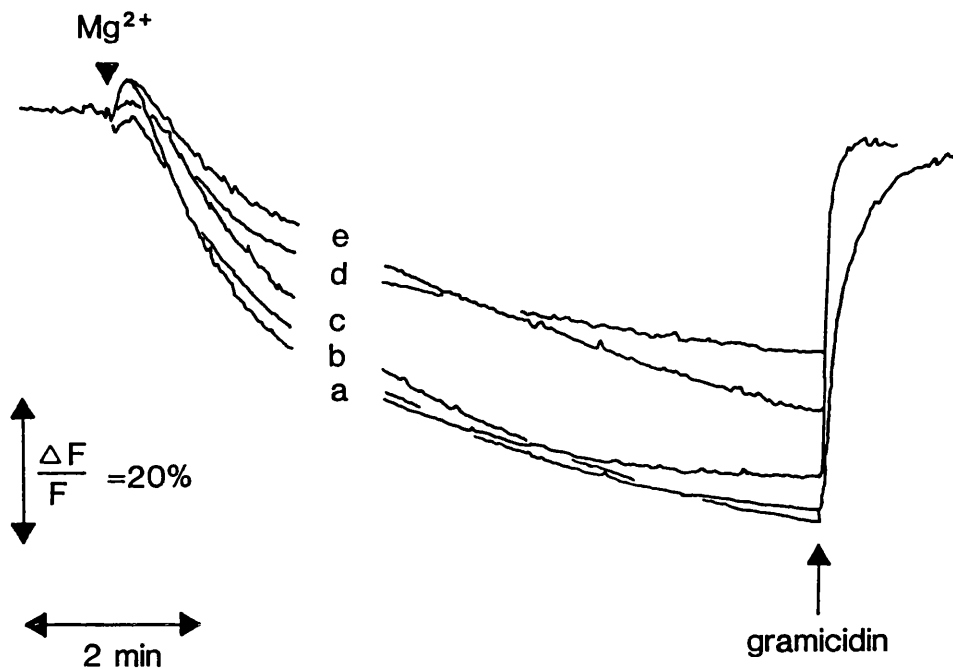
remain even when high concentrations of EDTA were used. This method was therefore not pursued further. In any case, the results from chapters 3 and 4 question whether it would be possible to resolve electrically-coupled  $\text{Na}^+$  and  $\text{H}^+$  fluxes from antiport-driven fluxes by using valinomycin.

Because of this, the approach of looking at the effect of  $\text{Na}^+$  on pH gradients generated by the ATPase without depleting the substrate was tried. As the tonoplast ATPase is insensitive to cations (Walker and Leigh 1981a; Poole et al. 1984; Bennett et al. 1984) any effects on the pH gradient must be due to secondary transport. The  $\text{H}^+$ -ATPase was used to generate a pH gradient, which was monitored with quinacrine fluorescence. On addition of 50 mM  $\text{Na}_2\text{SO}_4$  there was a partial recovery of quinacrine fluorescence (Fig. 5.2).



**Fig. 5.2.** Collapse of ATP-dependent pH gradient in tonoplast vesicles by  $\text{Na}_2\text{SO}_4$ , measured with quinacrine fluorescence. The reaction mixture consisted of tonoplast vesicles in 0.25 M glycerol, 50 mM KCl, 0.3 mM EGTA, 1.5 mM ATP-BTP, 5  $\mu\text{M}$  quinacrine, 5 mM BTP-MES pH 7.5. The reaction was started by the addition of 1.5 mM  $\text{MgSO}_4$ . Sodium sulphate was added as indicated. Gramicidin was added to a concentration of 2.5  $\mu\text{g ml}^{-1}$  to collapse the pH gradient.

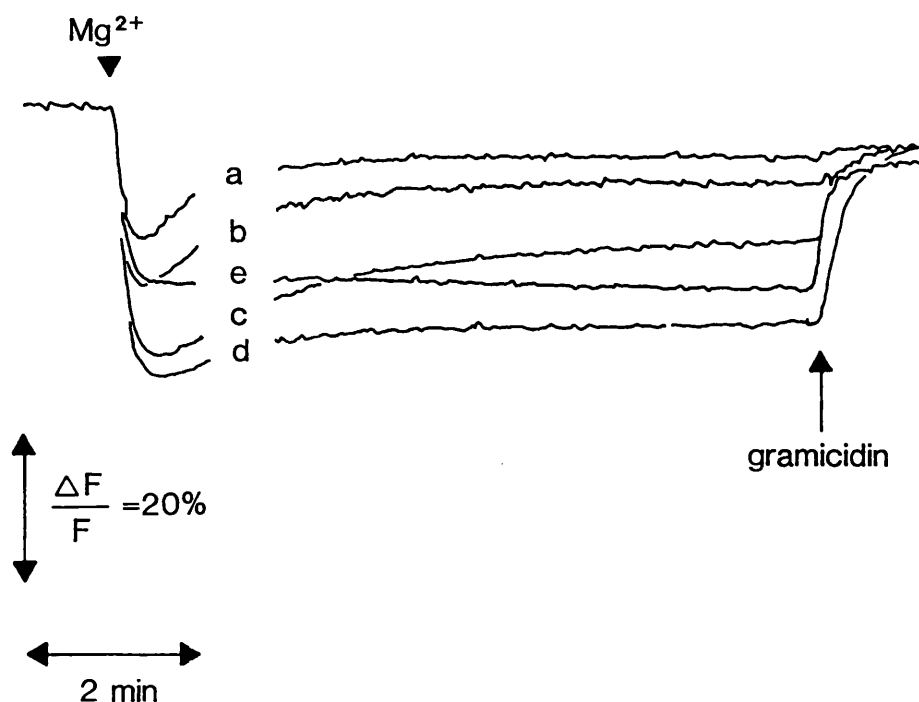
This apparent effect of  $\text{Na}^+$  on the pH gradient was examined further by looking at the ability of the ATPase to generate a pH gradient in the presence of different cation chlorides (Fig. 5.3). The initial rate of quinacrine fluorescence quenching varied according to the chloride present and was in the order  $\text{K} > \text{choline} > \text{BTP} > \text{Na} > \text{Tris}$ . The extent of pH gradient formation was smallest with Tris and Na, with the other cations all giving similar extents of pH gradient formation to each other.



**Fig. 5.3.** ATP-dependent pH gradients in tonoplast vesicles in the presence of different cations, measured with quinacrine fluorescence. The reaction mixture consisted of tonoplast vesicles in 0.25 M glycerol, 0.3 mM EGTA, 1.5 mM ATP-BTP, 5  $\mu\text{M}$  quinacrine, 5 mM BTP-MES pH 8.0 and 50 mM chloride as the K salt (a), the choline salt (b), the BTP salt (c), the Tris salt (d), or the Na salt (e). The reaction was started by the addition of 1.5 mM  $\text{MgSO}_4$ . Gramicidin was added to a concentration of 2.5  $\mu\text{g ml}^{-1}$  at the end of each assay to collapse the pH gradient.

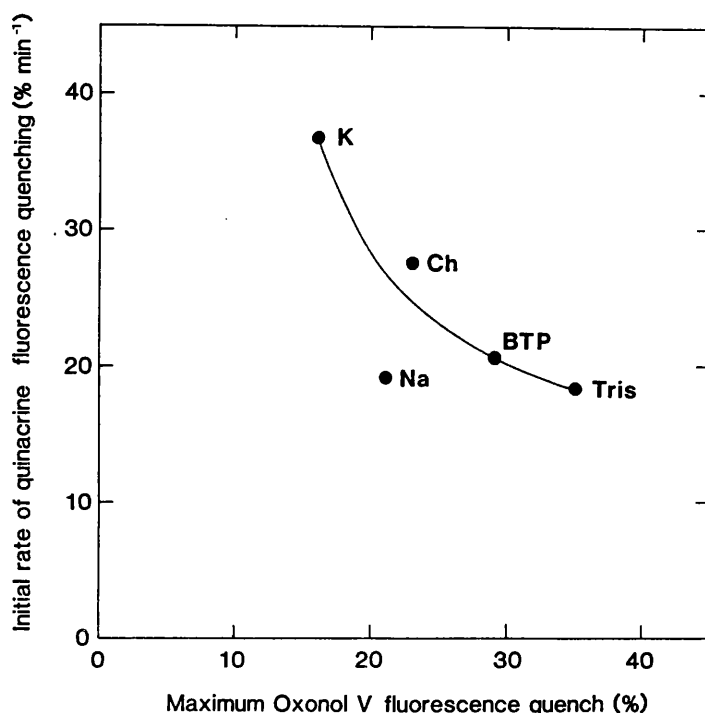
The effect of the different cations on the membrane potential generated by the ATPase was monitored by Oxonol V fluorescence.

The fluorescence of Oxonol V is quenched by a positive membrane potential. The membrane potential reported by this probe varied greatly according to the cation present (Fig. 5.4). The maximum quench of fluorescence was in the order Tris > BTP > choline > Na > K. When the initial rate of pH gradient formation was plotted against the quench of Oxonol V fluorescence, an inverse relationship was found (Fig. 5.5). Larger membrane potentials were associated with smaller rates of pH gradient formation. This shows that the effects of the different cations on the pH gradient and membrane potential were interdependent. These results raise the possibility that the effects of different cations on the initial rate of pH gradient formation were caused by the effect on the membrane potential, rather than a direct effect on the pH gradient. This



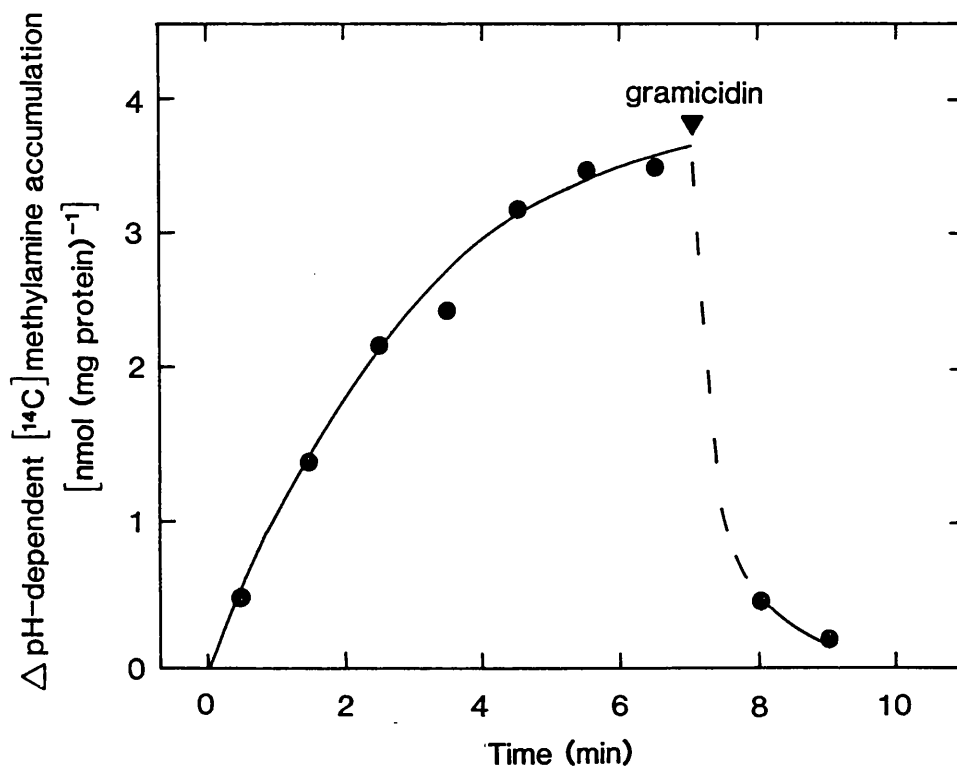
**Fig. 5.4.** ATP-dependent membrane potentials in tonoplast vesicles in the presence of different cations, measured with Oxonol V fluorescence. The reaction mixture consisted of tonoplast vesicles in 0.25 M glycerol, 0.3 mM EGTA, 1.5 mM ATP-BTP, 1  $\mu$ M Oxonol V, 5 mM BTP-MES pH 8.0 and 50 mM chloride as the K salt (a), the Na salt (e), the choline salt (b), the BTP salt (c) or the Tris salt (d). The reaction was started by the addition of 1.5 mM  $MgSO_4$ . Gramicidin was added to a concentration of 2.5  $\mu$ g  $ml^{-1}$  at the end of each assay to collapse the membrane potential.

questions whether monitoring the effect of  $\text{Na}^+$  on pH gradients generated by the ATPase can be used to look for a  $\text{Na}^+/\text{H}^+$  antiport.



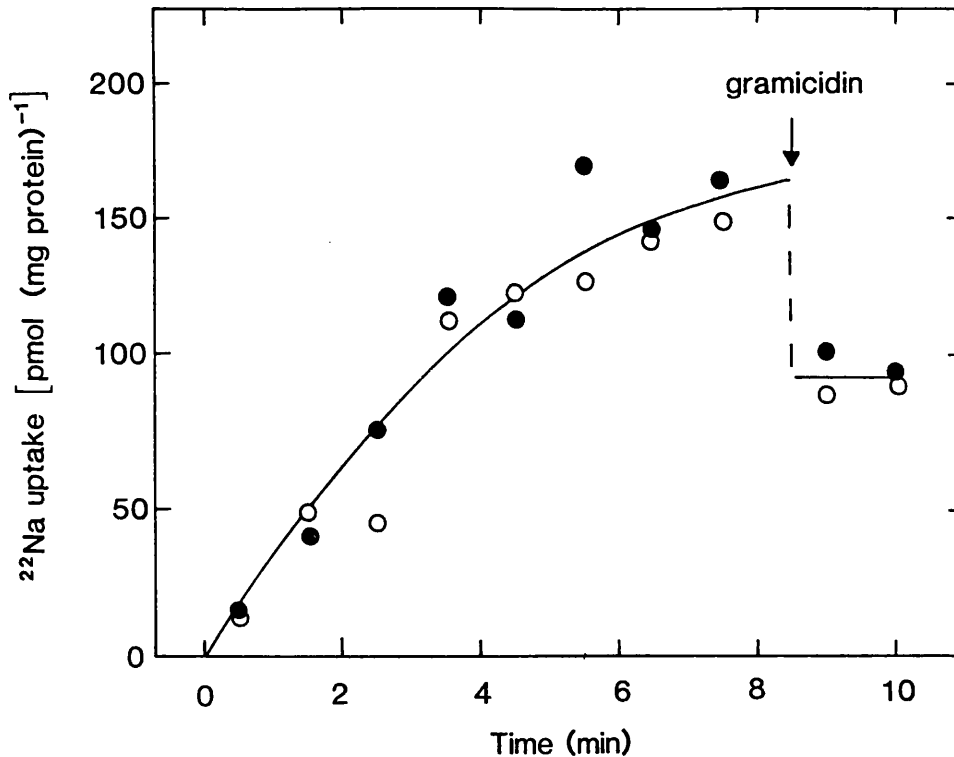
**Fig. 5.5.** Plot of initial rate of quinacrine fluorescence quenching against maximum Oxonol V fluorescence. Data obtained from the experiments shown in Figs. 5.3 and 5.4.

**5.3.2 Direct measurements of  $\text{Na}^+$  uptake in response to pH gradients generated by the  $\text{H}^+$ -ATPase.** In an attempt to settle the question of whether  $\text{Na}^+/\text{H}^+$  antiport activity was present, it was decided to measure  $\text{Na}^+$  transport directly in response to pH gradients generated by the ATPase. To determine whether the internal volume of the population of vesicles that are able to pump  $\text{H}^+$  and generate a pH gradient would be sufficient to see any pH gradient-dependent Na uptake, pH gradient formation was monitored using uptake of [<sup>14</sup>C]methylamine. No difficulties were encountered in using this probe to measure the pH gradient under these conditions and the accumulation of [<sup>14</sup>C]methylamine indicated formation of a pH gradient by the ATPase (Fig. 5.6).



**Fig. 5.6.** Accumulation of [<sup>14</sup>C]methylamine by tonoplast vesicles in response to an ATP-dependent pH gradient. The reaction mixture (1.0 ml) consisted of tonoplast vesicles (0.1 mg protein) in 0.3 M glycerol, 50 mM KCl, 3 mM ATP-BTP, 5 mM MgSO<sub>4</sub>, 0.3 mM EGTA, 20 μM [<sup>14</sup>C]methylamine and 5 mM BTP-MES pH 7.5. Reaction was started at t=0 by addition of vesicles. At intervals 100-μl aliquots were removed and the vesicles filtered on WCN cellulose nitrate membrane filters and washed as described in 3.2.2. The wash medium contained 0.25 M glycerol, 0.1 mM NaCl and 5 mM BTP-MES pH 7.5. At t=7 min 2.5 μg ml<sup>-1</sup> gramicidin was added to collapse the pH gradient.

Uptake of <sup>22</sup>Na was found to be independent of ATP-generated pH gradients (Fig. 5.7). It appeared that Na<sup>+</sup> was simply equilibrating across the membrane. The partial release of Na<sup>+</sup> that was seen on addition of gramicidin was not due to the collapse of the pH gradient as this was also seen in the control. This release of Na<sup>+</sup> probably happened during the filter wash procedure; on washing away the external Na<sup>+</sup>, internal Na<sup>+</sup> would flow out down its concentration gradient through the gramicidin pores in the membrane.

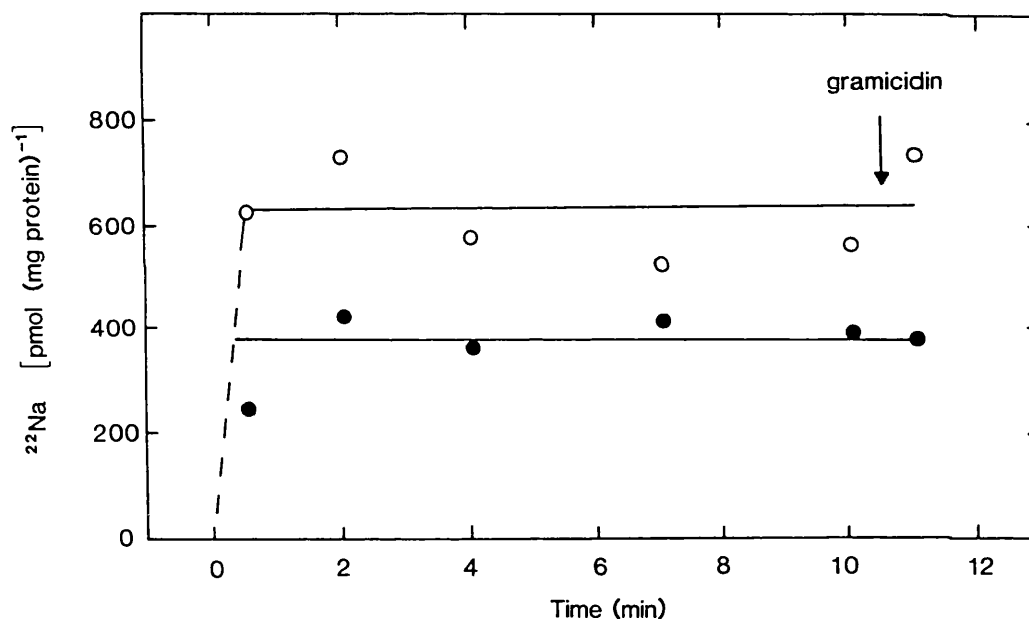


**Fig. 5.7.** Accumulation of  $^{22}\text{Na}$  by tonoplast vesicles in the presence (●) or absence (○) of an ATP-dependent pH gradient. The reaction mixture (1.0 ml) consisted of tonoplast vesicles (0.3 mg protein) in 1.0 ml 0.3 M glycerol, 50 mM KCl, 1.5 mM ATP-BTP, 0.3 mM EGTA, 0.1 mM  $^{22}\text{NaCl}$  and 5 mM BTP-MES pH 7.5 with (●) or without (○) 1.5 mM  $\text{MgSO}_4$ . Reaction was started at  $t=0$  by addition of vesicles. At intervals 100- $\mu\text{l}$  aliquots were removed and the vesicles filtered on WCN cellulose nitrate membrane filters and washed as described in 3.2.5. The wash medium contained 0.25 M glycerol and 5 mM BTP-MES pH 7.5. At  $t=8.5$  min  $2.5 \mu\text{g ml}^{-1}$  gramicidin was added to collapse  $\text{H}^+$  and  $\text{Na}^+$  gradients.

The possibility that this negative result was due to using an insufficient quantity of vesicles so that the internal volume of the vesicles loaded onto each filter was too small to enable pH gradient-dependent  $^{22}\text{Na}$  uptake to be seen was tested. However, attempts to use larger quantities of vesicles were not successful. The cellulose nitrate membrane filters used appeared to be blocked by the large amount of vesicles. Very erratic results were obtained



and no effect of the pH gradient on Na<sup>+</sup> uptake by the vesicles was seen (results not shown). To get around this technical problem glass fibre filters were tried. A high vesicle protein concentration was used (1.0 mg in 1.0 ml), together with 5 mM ATP to prevent substrate depletion. In this experiment, the vesicles were allowed to build up a pH gradient before the labelled NaCl was added to maximise any pH gradient-dependent <sup>22</sup>Na uptake. The vesicles used in this experiment were isolated from beets that had been watered with 25 mM NaCl. However, no ATP-dependent <sup>22</sup>Na uptake was observed (Fig. 5.8).



**Fig. 5.8.** Accumulation of <sup>22</sup>Na by tonoplast vesicles in the presence (●) or absence (○) of an ATP-dependent pH gradient. The reaction mixture (1.0 ml) consisted of tonoplast vesicles (1.0 mg protein) in 0.3 M glycerol, 50 mM KCl, 5 mM MgSO<sub>4</sub>, 0.3 mM EGTA, and 5 mM BTP-MES pH 7.5 with (●) or without (○) 5 mM ATP-BTP. Reaction was started by addition of vesicles 5 min before an aliquot of <sup>22</sup>NaCl was added at t=0 to give a NaCl concentration of 0.1 mM. At intervals 170- $\mu$ l aliquots were removed and the vesicles filtered on glass fibre filters and washed three times with 2 ml wash medium (0.25 M glycerol, 0.1 mM NaCl, 5 mM BTP-MES pH 7.5). At t=10.5 min 5.0  $\mu$ g ml<sup>-1</sup> gramicidin was added to collapse H<sup>+</sup> and Na<sup>+</sup> gradients.

#### 5.4 Discussion

The method of using the ATPase to generate a pH gradient, removing the substrate, and observing the effect of  $\text{Na}^+$  on the collapse of the pH gradient has been used by other workers to study  $\text{Na}^+/\text{H}^+$  exchange at the tonoplast. However, this method would appear not to have any advantages over pH jumps, due to the problem of resolving electrically-coupled fluxes from antiport-driven fluxes. This method was therefore not pursued further.

It was expected that the problems with the above approach could be avoided by looking at the effect of  $\text{Na}^+$  on pH gradients generated by the ATPase in the presence of continued  $\text{H}^+$  pumping by the ATPase. The presence of 50 mM  $\text{Cl}^-$ , a permeant anion, would be expected to keep the membrane potential close to zero under these conditions (Pope and Leigh 1987) so that any effects on the pH gradient would be direct, rather than due to changes in the relative contributions that the pH gradient and the membrane potential make to the protonmotive force. It was found that addition of  $\text{Na}^+$  led to a partial collapse of the pH gradient under these conditions (Fig. 5.2).

However, cations other than  $\text{Na}^+$  showed effects on the rate of pH gradient formation, compared with a  $\text{K}^+$  control. The initial rate of pH gradient formation was inversely related to the membrane potential. It is not clear from these results whether the effect of varying the cation on the membrane potential is a cause or an effect of the change on the rate of pH gradient formation. These results show that it cannot be assumed that the presence of 50 mM  $\text{Cl}^-$  will necessarily keep the membrane potential close to 0 mV. This means

it cannot be assumed that any effect of  $\text{Na}^+$  on the pH gradient must be due to a direct effect on the pH gradient via an antiport.

Therefore direct measurements of  $^{22}\text{Na}$  uptake in response to pH gradients generated by the  $\text{H}^+$ -ATPase are required to find out if there is a  $\text{Na}^+/\text{H}^+$  antiport present. Any  $^{22}\text{Na}$  uptake driven by the pH gradient in these conditions would have to be driven by a  $\text{Na}^+/\text{H}^+$  antiport. However, no evidence for ATP-dependent  $^{22}\text{Na}$  uptake was found. It is concluded that the effect of  $\text{Na}^+$  on the pH gradient generated by the ATPase is therefore an effect caused by an increase in the membrane potential. This may be due to a  $\text{Na}^+$  diffusion potential making the membrane potential more positive or possibly to  $\text{Na}^+$  reducing the  $\text{Cl}^-$  conductance of the vesicles so that chloride is less able to dissipate the membrane potential.

## **CHAPTER 6: GENERAL DISCUSSION**

### **6.1 Tonoplast Vesicle Preparation**

The experiments on tonoplast vesicle preparation (Chapter 2) confirmed previous observations (Bennett et al. 1984; Poole et al. 1984; Rea and Poole 1985), which indicated that tonoplast vesicles that are essentially free of contaminating membranes can be prepared from tissue homogenates of red beet. The work described in this thesis has extended these observations by taking into account the unsuitability of azide-sensitive ATPase as a mitochondrial marker in KI-washed membranes. These experiments also showed that a KI wash should not be used in the preparation of tonoplast as recoveries of marker enzymes associated with the tonoplast were reduced, but a resuspension medium wash should be used instead. It seems that the KI wash was used in published procedures for the preparation of tonoplast from red beet because this had been used in plasma membrane preparations, rather than because of any demonstrated benefits in tonoplast vesicle isolation. It was also of interest that omission of a microsomal wash step led to contamination of the preparation with plasma membrane. Pope and Leigh (1988b) found that replacing the glycerol in the resuspension medium with 0.5 M sorbitol led to tonoplast preparations with substantially reduced purity. This illustrates that small changes in the vesicle isolation procedure can have substantial effects on the purity of the vesicle preparation. If any modifications are made to a proven vesicle isolation procedure, then the purity of the preparation should be checked before assuming that the modifications have had no effect.

## 6.2 Problems Associated with the Use of pH Jumps to Measure

### Na<sup>+</sup>/H<sup>+</sup> Exchange

Experiments conducted using the pH jump technique revealed a number of serious problems with using this method to measure Na<sup>+</sup>/H<sup>+</sup> exchange. When pH jumps were performed using the media used by Blumwald and Poole (1985a), it was found that the changes in acridine orange fluorescence on addition of vesicles and the subsequent addition of Na<sub>2</sub>SO<sub>4</sub> were independent of the pH gradient. As all the observations made by Blumwald and Poole (1985a; 1987; Blumwald et al. 1987) used acridine orange as the pH gradient probe, the results presented here must cast some doubt on their interpretation. These pH gradient-independent changes in acridine orange fluorescence, probably caused by binding of the probe to vesicles and its displacement by cations, were dependent on the media used for the pH jump experiments. Under modified conditions (3.3.2) these problems were not seen and measurements of the pH gradient made with [<sup>14</sup>C]methylamine confirmed the results obtained with acridine orange. This shows that the behaviour of a pH probe should be checked under the exact conditions under which it is to be used, rather than relying on measurements made under different conditions.

It was found that Na<sup>+</sup> collapsed the pH gradient in tonoplast vesicles in the presence of a -120 mV K<sup>+</sup>/valinomycin membrane potential clamp and this effect showed Michaelis-Menten kinetics. However, this was also observed in phosphatidylcholine liposomes. Far from simply clamping the membrane potential to avoid electrically-coupled Na<sup>+</sup>/H<sup>+</sup> exchange, valinomycin actually increased electrically-coupled Na<sup>+</sup>/H<sup>+</sup> exchange when used at μM

concentrations. This effect was non-specific, independent of its role as a  $K^+$  ionophore, due to the high concentrations used. Valinomycin, at a concentration of 5  $\mu M$ , was able to increase the rate of  $Na^+$  transport into liposomes in response to a negative  $H^+$  diffusion potential resulting from a pH jump. This depolarised the membrane potential on which the pH gradient depended for stability, causing the pH gradient to collapse. However, even at much lower concentrations it was impossible to clamp the membrane potential in liposomes in the presence of  $Na^+$  at -120 mV. These findings are consistent with a number of reports in the literature about non-specific effects of valinomycin at  $\mu M$  concentrations (Krulwich 1983; Garcia et al. 1984; LaBelle 1984; Pope and Leigh 1987; Woolley et al. 1987). Garcia et al. (1984) found that when a  $K^+$ /valinomycin diffusion potential was imposed on liposomes to generate a pH gradient, addition of Na salts led to a collapse of the pH gradient when high concentrations of valinomycin were used. The results presented in this thesis (3.3.3; 4.3.1) are consistent with these findings and emphasise the problems inherent with using artificially-imposed pH gradients to measure  $Na^+/H^+$  antiport activity.

In pH jump experiments where no attempt was made to clamp the membrane potential, electrically-coupled  $Na^+/H^+$  exchange was observed, though at a lower rate than when  $\mu M$  concentrations of valinomycin were present. In the absence of valinomycin, the  $Na^+/H^+$  exchange properties of the tonoplast were like those of liposomes. Uptake of  $^{22}Na$  driven by a  $H^+$  diffusion potential was observed when a pH jump was imposed on liposomes under these conditions. Rea et al. (1987b) observed pH jump dependent uptake in tonoplast vesicles and ascribed it to a  $Na^+/H^+$  antiport. The

results obtained with liposomes in this thesis suggest that this could be accounted for by H<sup>+</sup> diffusion potential driven uptake.

The experimental studies discussed above show that pH jumps cannot be relied upon as a method for characterising a Na<sup>+</sup>/H<sup>+</sup> antiport. This conclusion is supported by thermodynamic considerations. Blackford et al. (1988) argued that pH jump experiments in tonoplast vesicles leaky to H<sup>+</sup> cannot be used to determine the stoichiometry of the tonoplast Ca<sup>2+</sup>/H<sup>+</sup> antiport because H<sup>+</sup> will be at equilibrium. Their argument will be applied here to the question of whether a Na<sup>+</sup>/H<sup>+</sup> antiport can be successfully studied in vesicles leaky to H<sup>+</sup> in pH jump experiments. The free energy equation that describes the operation of an electroneutral Na<sup>+</sup>/H<sup>+</sup> antiport is as follows:

$$\Delta G = 60(\text{pH}_i - \text{pH}_o) + 60(\text{pNa}_o - \text{pNa}_i)$$

where  $\Delta G$  is the free energy change of the antiport in mV (negative for the pH gradient driving accumulation of Na, positive for efflux of Na driving pH gradient formation), pNa is analogous to pH, and i and o refer to the compartments inside and outside the vesicle. On substitution of the condition for H<sup>+</sup> being at equilibrium ( $\Delta\psi = 60[\text{pH}_i - \text{pH}_o]$ ), where  $\Delta\psi$  is the membrane potential, the above equation reduces as follows:

$$\Delta G = \Delta\psi + 60(\text{pNa}_o - \text{pNa}_i)$$

In other words, operation of the antiport is dependent on the membrane potential and the imposed Na<sup>+</sup> gradient but independent of the pH gradient. These are the same conditions that would be

expected of  $\text{Na}^+$  uptake through a channel or leak in the membrane in response to the membrane potential. It follows that experiments in vesicles leaky to  $\text{H}^+$  where uptake of  $^{22}\text{Na}$  is measured will not distinguish between electrophoretic uptake of  $\text{Na}^+$  and uptake of  $\text{Na}^+$  via a  $\text{Na}^+/\text{H}^+$  antiport. This conclusion applies to vesicles with a 2 unit pH gradient with a membrane potential of  $-120$  mV, whether this results from a  $\text{H}^+$  diffusion potential or is imposed with  $\text{K}^+/\text{valinomycin}$ . Even if it were possible to clamp at  $-120$  mV in the presence of  $\text{Na}^+$ , these thermodynamic considerations show that this assay would not be suitable for making direct measurements of  $\text{Na}^+$  transport. The limitations of the pH jump technique discussed here for making direct measurements of  $\text{Na}^+$  transport would apply to any vesicle system that is not tightly sealed to  $\text{H}^+$ , so that  $\text{H}^+$  is at equilibrium.

The conclusions about the unsuitability of pH jumps for studying  $\text{Na}^+/\text{H}^+$  exchange described in Chapters 3 and 4 were derived from experimental studies. They are supported by the thermodynamic argument outlined above. The main conclusion from the work on pH jumps is that this technique is unsuitable for attempting to assay a  $\text{Na}^+/\text{H}^+$  antiport. This raises the question of whether a  $\text{Na}^+/\text{H}^+$  antiport is present at the tonoplast of red beet.

It might be possible to look for transient antiport-mediated  $^{22}\text{Na}$  uptake in vesicles clamped at  $0$  mV as although the pH gradient collapses rapidly in this situation, equilibrium is not reached until the pH gradient has collapsed completely. Therefore the collapsing pH gradient would be able to drive uptake of  $\text{Na}^+$ . However, it was considered more satisfactory to break from the pH jump technique altogether and to use pH gradients generated by the



ATPase. A pH gradient generated by the ATPase is unstable thermodynamically but stable kinetically as the H<sup>+</sup> pumping matches the outward leak of H<sup>+</sup>. Therefore the pH gradient generated by the ATPase would be able to provide a driving force for a Na<sup>+</sup>/H<sup>+</sup> antiport.

### 6.3 Is There a Na<sup>+</sup>/H<sup>+</sup> Antiport at the Tonoplast of Red Beet?

Measurement of the effect of Na<sup>+</sup> on the pH gradient generated by the ATPase was inconclusive (5.3.1). Although Na<sup>+</sup> led to a partial collapse of the pH gradient in tonoplast vesicles and reduced the rate of ATP-dependent pH gradient formation compared with a K<sup>+</sup> control, other cations reduced the rate of pH gradient formation by the ATPase. Also, this was associated with an increase in membrane potential, so it is not clear whether the decrease in pH gradient was a cause or consequence of the increase in membrane potential. However, when Na<sup>+</sup> transport was studied directly by using <sup>22</sup>Na no ATP-generated pH gradient-dependent uptake was found. No problems were encountered in measuring [<sup>14</sup>C]methylamine accumulation in response to the pH gradient generated by the ATPase, which strongly suggests that if there was any pH gradient-driven accumulation of Na<sup>+</sup> then the <sup>22</sup>Na uptake assays would have detected it. It appears that a Na<sup>+</sup>/H<sup>+</sup> antiport is not present at the tonoplast of red beet storage roots. Assays of this kind have been used successfully to measure a Na<sup>+</sup>/H<sup>+</sup> antiport at the tonoplast of barley (Garborino and DuPont 1989). Similarly, pH gradients generated by the ATPase in red beet tonoplast vesicles will drive uptake of <sup>45</sup>Ca via a Ca<sup>2+</sup>/H<sup>+</sup> antiport (Blackford et al. 1988; 1989). This supports the conclusion that the result seen here does indicate that the red beet tonoplast vesicles used do not possess a Na<sup>+</sup>/H<sup>+</sup> antiport.

Garborino and DuPont (1988) reported that the tonoplast of barley contained a  $\text{Na}^+/\text{H}^+$  antiport that was induced by treatment of the plants with NaCl and Blumwald and Poole (1985a) grew their beets with added NaCl. In sugar beet suspension culture cells, Blumwald and Poole (1987) reported that growing cells with added NaCl induced the  $\text{Na}^+/\text{H}^+$  antiporter above a constitutive level. This raises the possibility that the discrepancy between the conclusions of the work described in this thesis and those of other workers could be accounted for by the presence or absence of an inducible  $\text{Na}^+/\text{H}^+$  antiport. However, this seems unlikely for the following reasons. When pH jumps were performed as described by Blumwald and Poole (1985a), addition of  $\text{Na}_2\text{SO}_4$  caused a large recovery of fluorescence (3.3.1), as observed by Blumwald and Poole (1985a). It is the conclusions from the control experiments reported in 3.3.1 that lead to the different conclusions from those of Blumwald and Poole (1985a). In experiments where the ATPase was used to generate a pH gradient, no pH gradient-dependent  $\text{Na}^+$  uptake was seen even when tonoplast vesicles were prepared from NaCl-treated beets (5.3.2).

Rea et al. (1987b) reported on the basis of pH jump experiments that washing discs of red beet tissue in aerated water induced a  $\text{Na}^+/\text{H}^+$  antiport. However, these results must be interpreted with caution as pH jump experiments alone will not distinguish between antiport-coupled fluxes and electrically-coupled fluxes. These results could be accounted for simply by an increase in the leakiness of the membrane to  $\text{Na}^+$ .

The conclusion that the tonoplast of red beet does not contain a  $\text{Na}^+/\text{H}^+$  antiport is not in conflict with the available evidence

about the compartmentation of  $\text{Na}^+$  between cytoplasm and vacuole in fresh red beet storage root. Macklon (1975), using tracer efflux analysis on fresh beet tissue, obtained data that give cytoplasmic and vacuolar  $\text{Na}^+$  concentrations of 54 and 19 mM respectively, assuming that the cytoplasmic and vacuolar compartments occupy 5% and 70% of the disc volume (Pitman 1963). Although most of the  $\text{Na}^+$  in the cell will be in the vacuole because of its large volume, there would seem to be no reason to require that a  $\text{Na}^+$  transport mechanism that establishes a substantial concentration gradient need be present. In red beet tissue that has been washed for several days in aerated water, the available evidence is somewhat conflicting. Using tracer efflux analysis (Pitman 1963) obtained data that give cytoplasmic and vacuolar  $\text{Na}^+$  concentrations of 44 and 50 mM respectively, whereas the data of Macklon (1975) give values of 12 and 34 mM respectively.

In contrast, in barley there is consistent evidence for a concentration gradient of  $\text{Na}^+$  across the tonoplast (1.2) as well as evidence for a  $\text{Na}^+/\text{H}^+$  antiport. With a vacuolar  $\text{Na}^+$  accumulation ratio of between 7 and 8 (Jeschke and Stelter 1976),  $\text{Na}^+$  transport into the vacuole would need to be active. Garborino and DuPont (1989) found that a pH gradient generated by the ATPase in barley root tonoplast vesicles was able drive accumulation of  $^{22}\text{Na}$  against its concentration gradient, in contrast to the results obtained here in red beet. There is also in vivo evidence from  $^{23}\text{Na}$  and  $^{31}\text{P}$  NMR for a  $\text{Na}^+/\text{H}^+$  antiport in barley roots (Fan et al. 1989).

It is proposed that  $\text{Na}^+$  is distributed passively between the cytoplasm and vacuole in red beet storage root on the basis of the

available evidence. Passive transport of  $\text{Na}^+$  between cytoplasm and vacuole is presumably via ion channels which are able to conduct  $\text{Na}^+$  ions as well as other species. Such channels have been studied extensively in sugar beet (Coyaud et al. 1987; Hedrich and Neher 1987; Pantoja et al. 1989). In conclusion, there appears to be no clear evidence for a  $\text{Na}^+$  concentration gradient across the tonoplast of red beet and no good evidence for a  $\text{Na}^+/\text{H}^+$  antiport.

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