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`Chloride-Stimulated ATPase' Activity in Limonium vulgare Mill

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'Chloride-stimulated ATPase' activity in *Limonium vulgare* Mill.

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Limonium vulgare Mill. (sea lavender) is a saltmarsh plant in whose leaves are embedded multicellular glands for the excretion of salt. Physiological studies have shown that the basis of the salt secretion mechanism appears to be salt-inducible, ATP-powered pumping of chloride ions out of gland cells. We have been investigating a 'chloride-stimulated' ATPase activity of solubilized *Limonium* leaf microsomes, which we thought was almost certainly part of a chloride pump involved in salt secretion because the properties of the enzyme activity correlated so well with the characteristics of salt secretion. Methods of purifying this activity on hexyl agarose and by selective solubilization with low concentrations of neutral detergent are presented. These and other results have made it necessary to revise our understanding of earlier findings on the purification of the 'Cl^--stimulated' ATPase on Sepharose-*N*-caproyl galactosamine. Recent results indicate that the apparent 'stimulation' by chloride can be accounted for by an inhibition of salt-inducible ATPase activity by the 'control', non-Cl^-, monovalent anion, benzene sulphonate, adopted because it resembles chloride yet did not support salt-secretion. Our results demonstrate some of the dangers in interpreting measurements of ion-stimulated ATPase activity, and make an instructive 'cautionary tale'.

General

Anion-stimulated ATPase activity has been detected in various membrane fractions from different plants. Investigations of its characteristics have not revealed a simple, unified picture. For each preparation there are differences in the extent to which different anions stimulate ATPase hydrolysis, in the optimum pH for this process (two broad groups are recognizable, pH optima *ca.* 6.5 and *ca.* 8.5), and in the sensitivity of the anion-specific activity to various inhibitors (Hill & Hill 1973*a*; Rungie & Wiskich 1973; Lin *et al.* 1977; Grubmeyer & Spencer 1979; Cambraia & Hodges 1980; Hendrix & Pierce 1980; Walker & Leigh 1981; Auffret & Hanke 1981; Stout & Cleland 1982; Gradmann *et al.* this symposium). These differences are due at least partly to differences between different membrane types within the plant cell. Thus ATPase activity stimulated by anions has been detected in the F_1-ATPase of plant mitochondria (Grubmeyer & Spencer 1979), in tonoplast membranes prepared by using isolated vacuoles from certain tissues (Lin *et al.* 1977; Walker & Leigh 1981), and in microsomal fractions enriched in plasma membrane (Cambraia & Hodges 1980; Hendrix & Pierce 1980). Differences between tissues with different functions and differences between species might also contribute to the variation (see Lin *et al.* 1977). Inter-organ and interspecific differences are likely to be especially marked where the activity is part of a highly adaptive feature like a specialized salt gland. Varying conditions of assay also contribute, in this case less fundamentally, to reported differences between anion-specific ATPases. Membrane preparations are complex both ultrastructurally and biochemically. They consist of a continuum of all types and sizes of vesicle, resealed to varying degrees and with different in–out orientations. Cambraia &

Hodges (1980) have shown that differences in the anion–specific ATPase activity of two separate membrane fractions from oat roots were due entirely to this type of effect. Even a pure preparation of a single membrane type obtained from isolated organelles is biochemically highly complex, and each unique assemblage of lipids and other proteins will modify the characteristics of anion-specific ATPases. Finally the nature of the activity will depend on the experimental technique used to prepare and assay it. The salt-inducible Cl^--ATPase of microsomes from leaves of *Limonium vulgare* Mill. was assayed by release of P_i without adding detergent (Hill & Hill 1973*a*). By using a linked enzyme assay to monitor the release of ADP, this activity was only detected after treating the microsomes with detergent (Auffret & Hanke 1981).

In this light, the significance of many of the reported properties of anion-specific ATPase activity of membrane fractions is questionable. Some of the problems would be eliminated if individual anion-specific ATPase enzyme proteins could be purified to homogeneity, and this is one of our aims. However, we shall not make the mistake of assuming that the properties of a pure ATPase are the same *in vivo*. Taking a protein component of an ion pump out of the physically and chemically complex environment of its membrane is bound to affect its properties in ways that we cannot assess unless we already know its function *in vivo* and the characteristics of that function. The problem is that for almost all the anion-specific ATPases reported, their function is virtually unknown. We do not even know which of the many anions which stimulate activity they ever meet *in vivo*. Hendrix & Pierce (1980) detected anion-specific ATPase activity in a 'plasma membrane' fraction from pea epicotyls and pointed out that in this tissue K^+ uptake and the transmembrane electrical potential difference are both functions of the *anion* content of the bathing solution, but even limited correlations of this sort can rarely be made.

Limonium Cl^--ATPase

The salt-inducible Cl^--stimulated ATPase of *Limonium* was unique in that its function was clear and many of the characteristics of its activity *in vivo* can be predicted from the results of physiological experiments.

In a series of physiological experiments A. E. Hill, later in conjunction with B. S. Hill, established the nature of salt secretion by the glands at the surface of the leaves of this salt marsh plant. The results have been comprehensively reviewed (Hill & Hill 1973*b*; Hill & Hill 1976) and will be described in summary only.

(*a*) A number of observations together strongly suggest that an electrogenic chloride pump is solely responsible for driving salt through the glands.

(i) Secretion depended on the presence of Cl^- (only Br^- and I^- were anywhere near as effective) and not at all on the nature of the cation.

(ii) The electrical signal from active glands (secretory potential) was negative.

(iii) Under short-circuit conditions, transport of Cl^- predominated over that of the cation, resulting in a stable negative current out of the gland.

(iv) Measurements of Na^+ efflux over a range of clamp voltages showed that this process is passive.

(*b*) A number of observations together suggest that this electrogenic chloride pump drives Cl^- out of the gland cells at the plasma membrane, using ATPase hydrolysis as a source of energy.

(i) In voltage-clamp conditions there was a close dependence of the active fluxes of ions on the clamping current, and the active flux of Cl^- was affected differently from that of the

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cation. Uncoupling the two fluxes in this way would not be possible if ions move through the gland inside vesicles and are thereby isolated from electrical effects at the plasma membrane. Observation (*a*) (ii) is also difficult to explain by vesicular transport. The vesicles would have to be loaded with Na^+ as well as Cl^-, or the cation would not be transported, and so vesicular secretion should be electrically neutral.

(ii) The pH of the secreted fluid was neutral, a piece of evidence tending to support the idea that Cl^- efflux is *not* coupled to a proton gradient across the plasma membrane.

(iii) The effects of inhibitors, light–dark transitions and anaerobiosis on the short circuit current (see (*a*) (iii)) were consistent with a role for ATP in Cl^- transport.

This work led directly to the discovery of a Cl^--stimulated ATPase activity in microsomes prepared from *Limonium* leaves, and the characteristics of the induction of Cl^--specific ATPase activity after salt treatment of salt-free leaves were found to match those of the parallel induction of Cl^- secretion, which confirmed that the function of the Cl^--ATPase is salt excretion. Although salt-free plants showed greatly reduced levels of Cl^--ATPase activity, they grow extremely well and so it is unlikely that this enzyme is involved in any activity not directly related to salt excretion. Its salt-inducibility is a tremendous assest for us and is the diagnostic feature that we have used to distinguish it from any other *Limonium* ATPase. Its specialized function in salt excretion by a saltmarsh plant ensures that Cl^- must be the bulk anion handled by the enzyme *in vivo*. Its specificity for other anions is therefore largely irrelevant.

Attempts to purify *Limonium* Cl^--ATPase

'*The great tragedy of science – the slaying of a beautiful hypothesis by an ugly fact.*'

T. H. Huxley

In a bid to investigate whether the Cl^--ATPase was associated with plasma membrane, attempts were made to resolve *Limonium* leaf microsomes by sucrose density gradient centrifugation. These attempts failed because the membrane vesicles aggregated in clumps. Suspecting that this might be lectin-mediated, we screened the microsomal and supernatant fractions for haemagglutination activity, which was detected and turned out to be strongly inhibited by *N*-acetylgalactosamine (figure 1*a*), weakly inhibited by galactose and not inhibited by *N*-acetylglucosamine or glucose.

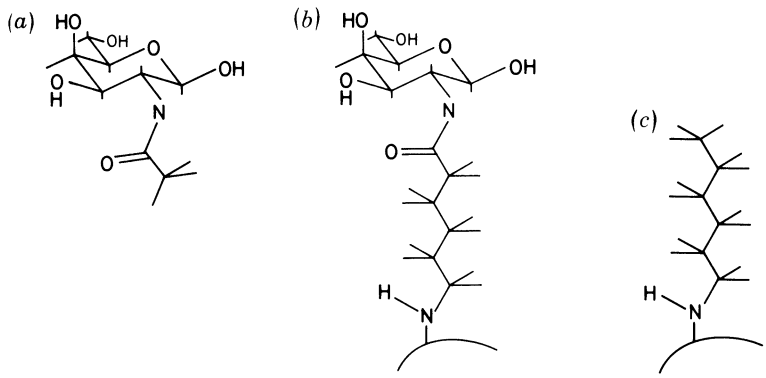

Figure 1. (*a*) Structure of *N*-acetyl galactosamine (GalNAc). (*b*) Structure of Sepharose-*N*-caproyl galactosamine (galactosamine-Sepharose). (*c*) Structure of hexyl agarose.

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During attempts to prevent the aggregation of microsomes by adding the inhibiting haptens, increases in 'Cl^--stimulated ATPase' activity were observed. *N*-acetylgalactosamine, the more potent inhibitor of haemagglutination, seemed to stimulate 'Cl^--stimulated ATPase' activity more strongly than galactose (table 1). Activation by this sugar suggested that the 'Cl^--stimulated ATPase' might possess a binding site specific for it, which we could exploit in

TABLE 1. SPECIFIC ACTIVITIES OF 'Cl^--STIMULATED' ATPASE IN *LIMONIUM* MICROSOMES

('Cl^--stimulated ATPase' activity was measured as the P_i released in 0.1 M NaCl in excess of that released in 0.1 M recrystallized sodium benzene sulphonate.)

	specific activity / μmol h^{-1} mg^{-1} protein	relative to microsomes (%)
(1) microsomes	0.26	—
microsomes + 2 mM Gal	0.43	160
microsomes + 1 mM GalNAc	0.54	210
(2) microsomes	0.38	—
microsomes + 2 mM Gal	0.50	130
microsomes + 0.4 mM GalNAc	0.69	180

affinity chromatography to purify the enzyme. Galactosamine-*N*-caproyl Sepharose (figure 1*b*) was chosen for the immobilized ligand and, after some adjustment of the conditions, we discovered that when microsomes solubilized in Triton X-100 (0.1 % by volume) in the presence of ATP were poured through this material and washed through with a small amount of buffer, 'Cl^--stimulated ATPase' activity could be eluted from the column in a solution of 0.2 M galactose in buffer. The resulting enzyme solution contained very little protein and so we appeared to have achieved a large increase in specific activity. There was an even more spectacular separation of 'Cl^--stimulated' activity from the 'basal ATPase', the activity in the presence of an equivalent concentration of benzene sulphonate. (In the early studies, sulphate was used as the control anion (Hill & Hill 1973*a*). However, because this ion is divalent it is not possible to match both its ionic strength and its osmotic activity with those of chloride. Benzene sulphonate, like sulphate, will not support salt secretion by *Limonium* leaf discs (Hill & Hill 1973*b*), but as a monovalent ion it more closely resembles chloride and so was adopted as the control anion in preference to sulphate (Hill & Hanke 1979)). The 'basal' activity had been sometimes as much as 90 % of the total ATPase of microsomes (Hill & Hanke 1979).

'*...beautiful hypothesis...*'

These results seemed to confirm the interaction between the 'Cl^--stimulated ATPase' and *N*-acetylgalactosamine, which raised the interesting possibility that the glycosylated components characteristics of the outer surface of the plasma membrane might activate or orientate, or both, ion pumps. This hypothesis would provide a neat explanation for the role of the small, species-specific glycoprotein subunit of all Na^+–K^+ATPases (Glynn & Karlish 1975), which in pig kidney tubule is found over the whole plasma membrane (Dibona & Mills 1979) and so is not solely a non-enzymic component of the exclusively basolateral ATPase. It might also explain the presence of sugar moieties and modified sugars in a number of compounds that alter the activity of plasma membrane ion pumps with high potency, e.g. the cardiac glycosides and fusicoccin.

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'...*ugly fact*...'

Before we could study the enzymology of the 'Cl^--stimulated ATPase' we had to develop a new assay. The existing method, measurement of P_i released after 1 h, was unsatisfactory in several respects:

(i) extra P_i was released from the substrate, ATP, by non-enzymic hydrolysis during P_i determination;

(ii) Triton X-100, in which we were now solubilizing microsomes, interfered in the determination of P_i to make the assay unworkable over part of its range (figure 2);

(iii) assay of P_i could not provide continuous monitoring of the progress of the reaction, or even measurements of the initial rate of reaction.

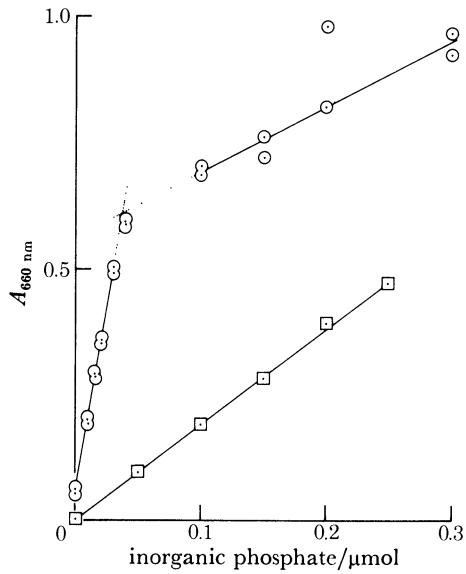

FIGURE 2. Standard curves for the assay of inorganic phosphate (Hill & Hill 1973*a*): ⊡, in water; ⊙, in 0.0083% (by volume) Triton X-100.

The linked-enzyme assay (Auffret & Hanke 1981) measures the rate of ADP release by coupling the production of this nucleotide to the oxidation of NADH, which can be monitored spectrophotometrically:

$$\mathrm{ATP} \overset{\text{ATPase}}{\rightleftharpoons} \mathrm{ADP} + P_i;$$

$$\mathrm{ADP} + \text{phosphoenolpyruvate} \overset{\text{pyruvate kinase}}{\rightleftharpoons} \text{pyruvate} + \mathrm{ATP};$$

$$\text{pyruvate} + \mathrm{NADH} \overset{\text{lactate dehydrogenase}}{\rightleftharpoons} \text{lactate} + \mathrm{NAD}.$$

ATP is regenerated, maintaining the initial concentration. The linked-enzyme assay was subject to none of the drawbacks listed for the original assay. A similar assay has been used by Warren *et al.* (1974) and by Møller *et al.* (1980) to measure ($Ca^{2+} + Mg^{2+}$)-stimulated ATPase activity in sarcoplasmic reticulum from rabbit skeletal muscle. We think that it should be more widely known and used. Even the latest work on plant ATPases (Stout & Cleland 1982) still uses an assay based on colorimetric determination of P_i.

Measurements of *initial* rates by the linked enzyme assay showed that there was *ca.* 10 times

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more 'Cl⁻-stimulated ATPase' activity in the microsomes than we had measured using the original assay (table 2). The full level of activity was not 'revealed' unless detergent was present, but 'Cl⁻-ATPase' activity then declined rapidly in the presence of detergent. This instability is probably a major reason why the full extent of detergent stimulation was never detected by using the P_i assay with its 60 min time point. These results emphasize the importance of measuring initial rates and of monitoring the reaction to check that rates are steady.

TABLE 2. SPECIFIC ACTIVITIES OF 'Cl⁻-STIMULATED ATPASE' ACTIVITY IN PREPARATIONS FROM *LIMONIUM* LEAVES

	specific activity (μmol h^{-1} mg^{-1} protein)	activity with Cl⁻ / activity with benzene sulphonate
microsomes by P_i assay	*ca.* 0.5	0.05–0.4
microsomes + 0.25 % Triton X-100 by linked enzyme assay	2–6.5	*ca* 0.8
partly purified enzyme	10–30	0.8–4.6

Both Triton X-100 and polyoxyethylene-9-lauryl ether, '9-L', at 0.25 % by volume in resuspended membranes gave comparable, strong stimulation up to the same level of activity (table 2). By comparison, the stimulation by *N*-acetylgalactosamine (table 1) can now be seen to be very slight and with the linked-enzyme assay this amino sugar, or galactose, did not further stimulate the initial rate in the presence of detergent in a reproducible manner.

This means that the purification achieved on galactosamine-Sepharose was almost certainly not due to affinity of the enzyme for the amino sugar. Recent results indicate that it was more likely to have been due to an affinity for the *N*-caproyl spacer arm. Thus a comparable separation from 'basal ATPase' can be achieved on hexyl agarose (figure 1*c*). When detergent-solubilized microsomes were poured through hexyl agarose, the unretarded material showed 'basal ATPase' activity only. Material held on the hexyl agarose could be released by increasing the ionic strength of the eluent. 'Cl⁻-stimulated ATPase' was eluted after a comparatively small increase in ionic strength, in fractions that did not coincide with the bulk protein (figure 3). The basis of this separation cannot be hydrophobic interaction alone, for which we would expect material to bind at high ionic strength and to be eluted as ionic strength is reduced.

Another curious aspect of the purification on galactosamine-Sepharose was that 'Cl⁻-stimulated ATPase' activity was detected in galactose eluates that ostensibly contained no detergent (but in practice would probably have been contaminated from that used to solubilize the microsomes). Pumps able to drive ions across membranes would be expected to be hydrophobic in nature and therefore insoluble in this kind of solution. However, we have recently been able to show that detergents such as 9-L at levels as low as 0.02 % will solubilize 60 % of the total 'Cl⁻-ATPase' from microsomes but less than 15 % of the total protein (figure 4). This selective solubilization gives the best separation from non-ATPase proteins of any method, although the separation from 'basal ATPase' activity is slight. Adding back phospholipid (100 μg ml^{-1} phosphatidyl choline) to the solubilized enzyme increased the 'Cl⁻-ATPase' activity. Loss of activity during purification, possibly due to removal of factors like phospholipids necessary for full activity, has been a recurring problem.

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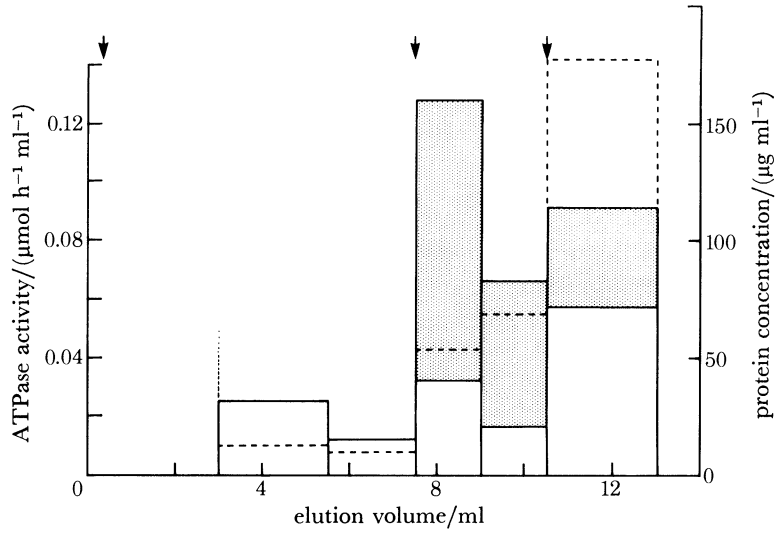

FIGURE 3. Partial purification of 'Cl⁻-stimulated ATPase' activity on hexyl agarose. *Limonium* leaf microsomes were solubilized by treatment with 9-L(1 g l^{-1}). The extract was applied to a *ca.* 1 ml column of hexyl agarose equilibrated with 2 mM EDTA, 30 mM TES, pH 7.0, 1 g l^{-1} 9-L solution. The column was then eluted with this buffer (first arrow) containing in addition increasing concentrations of sodium benzene sulphonate (second arrow, 50 mM; third arrow, 100 mM). The eluate was assayed for protein and ATPase activity by the linked-enzyme assay, with benzene sulphonate as control anion. Shaded columns, 'Cl⁻-ATPase'; open columns, 'basal ATPase'; broken line, protein, based on bovine serum albumin as standard.

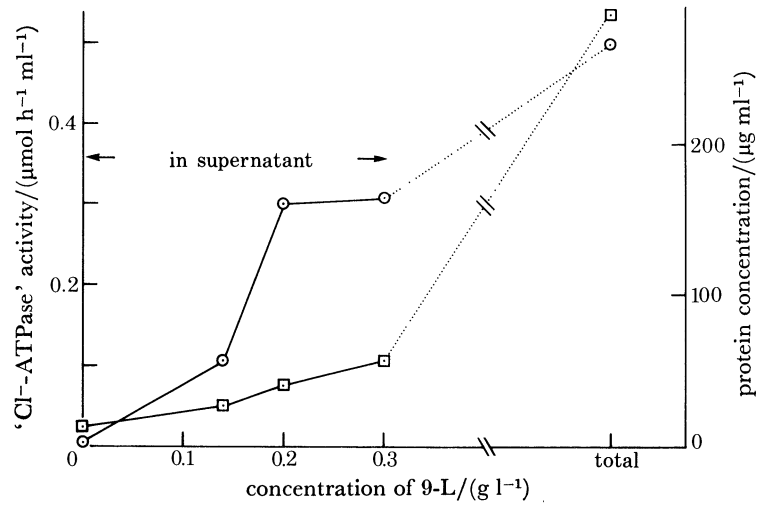

FIGURE 4. Selective solubilization of 'Cl⁻-stimulated ATPase' activity. *Limonium* leaf microsomes were diluted to *ca.* 300 μg ml^{-1} protein with glycerol (100 g l^{-1}), 30 mM TES, pH 7.0, 2 mM EDTA. Varying amounts of 9-L were added and the treated microsomes left on ice for 10 min. After centrifugation (1 h at 50000 *g*) the supernatant was assayed for protein and 'Cl⁻-stimulated ATPase' activity by the linked-enzyme assay, with benzene sulphonate as control anion. 100 μg ml^{-1} soybean phosphatidyl choline and 0.1 g l^{-1} 9-L were included in the ATPase assays. ⊙, 'Cl⁻-ATPase', ⊡, protein, based on bovine serum albumin as standard.

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Table 2 summarizes the progress so far in purifying the *Limonium* 'Cl⁻-stimulated ATPase' activity. Although the best specific activity that we can obtain is still quite low, our latest preparations are solubilized and show greatly reduced levels of 'basal ATPase'.

TABLE 3. CHARACTERISTICS OF THE 'Cl⁻-STIMULATED ATPASE' ACTIVITY IN DETERGENT-TREATED MICROSOMES FROM *LIMONIUM* LEAVES

('Cl⁻-stimulated ATPase' activity was measured by the linked enzyme assay as the ADP released in 0.12 M NaCl in excess of that released in 0.12 M recrystallized sodium benzene sulphonate.)

apparent K_m for ATP	0.1–0.2 mM
Cl^- stimulation	up to 0.13 M
pH optimum	6.3–6.4
sensitivity to 17 μM VO_4^{3-}	50 % inhibition in 0.5 mM ATP

PROPERTIES OF THE *LIMONIUM* Cl⁻-STIMULATED ATPASE

The enzymology of these latest preparations has not been studied. The results of work on the properties of 'Cl⁻-stimulated ATPase' activity in detergent-treated microsomes with the use of the linked enzyme assay are summarized in table 3 and discussed below.

(i) Since the concentration of ATP in the cytosol of illuminated protoplasts from green leaves of spinach is estimated at 0.13 mM (Stitt *et al.* 1980), the measured apparent K_m for ATP seemed to be of the right order.

(ii) Apparent stimulation by increasing concentrations of chloride was observed up to 0.13 M. The concentration of chloride in the xylem sap of a mangrove species growing in sea water and secreting salt through foliar salt glands was estimated to vary between 0.085 and 0.122 M (Atkinson *et al.* 1967), and so the affinity of the enzyme for Cl^- also appeared to be of the right order.

A POSTSCRIPT

...even more ugly facts!

Recent experimental results have shown that in spite of all these favourable correlations 'Cl⁻-stimulated ATPase' activity, measured by the linked-enzyme assay as the excess ADP released in 0.12 M NaCl over that released in 0.12 M sodium benzene sulphonate, is probably an artefact.

Until now we had not investigated the effect of ions other than chloride on this ATPase activity. Its presumed function in salt secretion made this information irrelevant. However, in recent experiments, the same concentration of acetate or sulphate was substituted for chloride and the same apparent stimulation was obtained. Indeed, by omitting the addition of chloride altogether we could obtain the same 'stimulation' over the 'basal ATPase', which is the activity measured in the presence of 0.12 M benzene sulphonate. The only reasonable interpretation of these results is that we have been measuring a benzene sulphonate-inhibited ATPase and *not* a chloride-stimulated ATPase.

None of our previous results using the linked enzyme assay disprove this interpretation, e.g. in studies of Cl^--dependency (Auffret & Hanke 1981) we were careful (!) to mix chloride and benzene sulphonate in varying proportions so as to maintain constant ionic strength.

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The significance of benzene sulphonate-inhibited ATPase is hard to assess. The ease with which the activity can be solubilized suggested that it might be the F_1-ATPase, but this is ruled out by its sensitivity to vanadate, plus the fact that the microsomes were prepared in the presence of EDTA. The most curious property of the benzene sulphonate-inhibited activity is its apparent salt-inducibility, in respect of which it correlates with salt secretion and with Cl^--stimulated ATPase activity as determined by P_i release using sulphate as control anion. We used this property, inducibility by salt, as a diagnostic feature for components of the ion pump of the salt gland and this explains why we did not suspect that the difference in activity in the presence of chloride and benzene sulphonate could be an artefact. The dangers of relying on a single diagnostic feature are now apparent. Another curious feature is that not all ATPase activity is benzene sulphonate-inhibited but only a specific fraction.

These recent findings do not invalidate the discovery and measurement of a salt-inducible, Cl^--stimulated ATPase in microsomes prepared from *Limonium* where sulphate and not benzene sulphonate was used as the control anion (Hill & Hill 1973*a*). This activity was detected by P_i release from ATP in the absence of detergent. It is, however, very curious that when the more sensitive linked-enzyme assay is used to measure ADP release from ATP, this activity cannot be detected in comparable membrane fractions from the same tissue, even when solubilized in neutral detergent. Instead, a detergent-stimulated, benzene sulphonate-inhibited activity increases on salt treatment. We do not yet rule out the possibility that these two activities, chloride-stimulated release of P_i from ATP in the absence of detergent and benzene sulphonate-inhibited release of ADP from ATP in the presence of detergent, are manifestations of the same ion-pumping mechanism.

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