Plant Physiol. (1984) 74, 545–548
0032-0889/84/74/0545/04/$01.00/0

H^+-ATPase Activity from Storage Tissue of *Beta vulgaris*[1]

II. H^+/ATP STOICHIOMETRY OF AN ANION-SENSITIVE H^+-ATPase

Received for publication June 30, 1983 and in revised form October 5, 1983

ALAN B. BENNETT*[2] AND ROGER M. SPANSWICK
Section of Plant Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

ABSTRACT

The H^+/ATP stoichiometry was determined for an anion-sensitive H^+-ATPase in membrane vesicles believed to be derived from tonoplast. Initial rates of proton influx were measured by monitoring the alkalinization of a weakly buffered medium (pH 6.13) following the addition of ATP to a suspension of membrane vesicles of *Beta vulgaris* L. Initial rates of ATP hydrolysis were measured in an assay where ATP hydrolysis is coupled to NADH oxidation and monitored spectrophotometrically (A_{340}) or by monitoring the release of ^{32}P from [γ-^{32}P]ATP. Inasmuch as this anion-sensitive H^+-ATPase is strongly inhibited by NO_3^-, initial rates of H^+ influx and ATP hydrolysis were measured in the absence and presence of NO_3^- to account for ATPase activity not involved in H^+ transport. The NO_3^--sensitive activities were calculated and used to estimate the ratio of H^+ transported to ATP hydrolyzed. These measurements resulted in an estimate of the H^+/ATP stoichiometry of 1.96 ± 0.14 suggesting that the actual stoichiometry is 2 H^+ transported per ATP hydrolyzed. When compared with the reported values of the electrochemical potential gradient for H^+ across the tonoplast measured *in vivo*, our result suggests that the H^+-ATPase does not operate near equilibrium but is regulated by cellular factors other than energy supply.

Membranes isolated from red beet (*Beta vulgaris* L.) have been shown to contain two H^+-translocating ATPases (5). In the accompanying paper (5), evidence was presented strongly supporting the proposal that the anion-sensitive H^+-ATPase associated with low density membranes is of tonoplast origin. Furthermore, these low density membranes are tightly sealed vesicles as evidenced by the large ionophore stimulation of ATPase activity and by the ability to measure the generation of ATP:Mg-dependent pH gradients. The extremely high ionophore stimulation of ATPase activity (>300%) in the low density membrane preparation suggested that a very high proportion, if not all, of the ATPase activity was present in sealed vesicles and coupled to H^+ transport. It could, therefore, be a useful preparation in estimating the stoichiometry of coupling between H^+ transport and ATP hydrolysis.

The determination of coupling stoichiometries for several ion-translocating ATPases has been a major goal in recent years (1, 12–14, 18, 22). A knowledge of the stoichiometry of coupling for Na^+ and K^+ transport by the Na^+/K^+-ATPase of animal cells is important in understanding the electrogenic properties of this ion-translocating ATPase (8). Similarly, a knowledge of the stoichiometry of coupling for H^+ and K^+ transport by the gastric H^+/K^+-ATPase is important in evaluating the energetic competence of this ATPase to sustain the large pH gradients observed *in vivo* (13, 14). The stoichiometry for H^+ transport by the plasma membrane H^+-ATPase of plant cells has also been of concern to plant cell electrophysiologists because this stoichiometry influences the maximal ion gradients sustainable across the plasma membrane (12, 18, 22). Estimates of the H^+/ATP stoichiometry of the plasma membrane H^+-ATPase of plant cells, based on electrophysiological evidence, indicate that 2 H^+ are transported per ATP hydrolyzed whereas in *Neurospora* this stoichiometry is apparently 1 (22). Relatively little attention has been paid to the energetics of H^+ transport at the tonoplast. However, recently the transport processes associated with this membrane have become accessible to biochemical characterization (5, 6, 10). In this report we look directly at the stoichiometry of coupling between H^+ transport and ATP hydrolysis in a tonoplast membrane fraction.

A model illustrating the coupling of H^+ transport to ATP hydrolysis by a H^+-ATPase is shown in Figure 1. As indicated in equation 1 (Fig. 1), the maximal electrochemical potential gradient for H^+ ($\Delta\bar{\mu}_{H^+}$)[3] that can be formed by the H^+-ATPase is a function of the free energy of hydrolysis of ATP (ΔG_{ATP}) and the stoichiometry of H^+ transported per ATP hydrolyzed (n). If one assumes that the H^+ pump is operating near equilibrium *in vivo*, then the H^+/ATP stoichiometry (n) can be estimated from a measurement of $\Delta\bar{\mu}_{H^+}$ across the membrane in question and a knowledge of ΔG_{ATP} in the cellular environment (1). Using a similar, thermodynamic, approach with isolated membrane vesicles is complicated by the high conductance of the isolated vesicle membranes to H^+ which prevents the development of maximal ion gradients *in vitro* (14).

In order to avoid problems associated with H^+ leaks in membrane vesicles, a kinetic rather than thermodynamic approach has been favored in estimating H^+/ATP stoichiometry of the gastric H^+/K^+-ATPase (13, 14). This kinetic method involves comparisons of the initial rates of proton influx with the initial rates of ATP hydrolysis. Under initial rate conditions, H^+ gradients are small and so the initial rates of H^+ transport should not be affected by H^+ leaks. This latter approach has been used here to estimate the H^+/ATP stoichiometry of the anion-sensitive H^+-ATPase from red beets, believed to be of tonoplast origin (5).

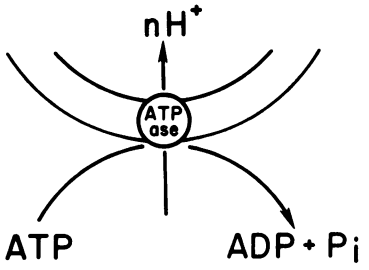
MATERIALS AND METHODS

Membrane Preparation. Microsomal membranes from red beet (*Beta vulgaris* L.) storage tissue were prepared and low density membrane vesicles collected from a 16/26% (w/w) su-

[1] Supported by United States Department of Agriculture Competitive Grants Program grant 81-CRCR-1-0758.

[2] Present address: Department of Vegetable Crops, University of California, Davis, CA 95616.

[3] Abbreviations: ΔG_{ATP}, free energy of ATP hydrolysis; $\Delta\bar{\mu}_{H^+}$, electrochemical potential gradient for H^+; BTP, (1,3-bis[tris(hydroxymethyl) methylamino] propane; $\Delta\psi$, membrane potential (mV).



$$\Delta G_{ATP} \geq n\,\Delta\bar{\mu}_{H^+} \quad (1)$$

FIG. 1. Schematic diagram illustrating the coupling of ATP hydrolysis to H^+ transport. Equation 1 expresses the quantitative relationship between the chemical (ATP hydrolysis) and osmotic (H^+ transport) reactions.

crose discontinuous gradient as described in the accompanying paper (5). This membrane fraction was diluted in 2 mM DTT, 5 mM Tris/Mes (pH 6.5), and was subsequently repelleted. The pellet was then resuspended in 250 mM sucrose, 0.5 mM $MgSO_4$, and 1 mM BTP/Mes (pH 6.13) at a concentration of 1.4 to 2.0 mg membrane protein/ml.

Proton Transport. Membrane vesicles (138 μl) were diluted in a stirred reaction cell at room temperature (approximately 23°C) with 862 μl of 250 mM sucrose, 0.5 mM $MgSO_4$, 1 mM BTP/Mes (pH 6.13), and either 58 mM KCl or 58 mM KNO_3. The pH of this vesicle suspension was then carefully adjusted to pH 6.13 with either 1 mM NaOH or HCl. Proton influx was initiated by the addition of 13 μl of 38 mM $MgCl_2$, 38 mM Na_2ATP with the pH adjusted to 6.13 with KOH. This gave final assay concentrations of 50 mM KCl or KNO_3, 1.0 mM Mg^{2+}, 0.5 mM ATP, and approximately 225 μg membrane protein/ml.

Proton loss from the vesicle medium was measured with a semimicro combination glass pH electrode (Microelectrodes Inc., model MI-410) attached to a Keithley 604 electrometer and the amplifier output recorded with an MFE model 2125 chart recorder. Proton loss was calibrated after each measurement by the addition of 10 μl of either 1 mM NaOH or 1 mM HCl. The response to added H^+ or OH^- for calibration indicated that the time required for mixing and electrode response was less than 1 s.

ATPase Assay. ATPase activity was determined by coupling ATP hydrolysis to NADH oxidation with P-enolpyruvate/pyruvate kinase and lactate dehydrogenase and monitoring NADH oxidation spectrophotometrically. Details of these coupling reactions are outlined by Auffret and Hanke (3). Membrane vesicles were diluted 5-fold with 250 mM sucrose, 0.5 mM $MgSO_4$, and 1 mM BTP/Mes (pH 6.13). A reaction mixture of 900 μl of 250 mM sucrose, 0.5 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.5 mM Na_2ATP, 5 units pyruvate kinase, 12 units lactate dehydrogenase, 0.42 mM P-enolpyruvate, 0.15 mM NADH, 1 mM BTP/Mes (pH 6.13), and either 50 mM KCl or KNO_3 was put into a 1.5 ml glass cuvette. The *A* at 340 nm was monitored with a Varian 634 spectrophotometer and recorded with an MFE model 2125 chart recorder. After recording a stable baseline *A* at 340 nm, the rection was initiated by the addition of 100 μl of the diluted membrane vesicles (30 to 50 μg membrane protein). Initial rates of NADH oxidation were calculated and equated with initial rates of ATP hydrolysis given that the coupling is 1 NADH oxidized/ATP hydrolyzed in this assay.

Alternatively, initial rates of ATPase activity were measured by monitoring the time course of ^{32}P release from [γ-^{32}P]ATP. Assays were set up exactly as for proton influx measurements (see previous section), except that the Na_2ATP, $MgCl_2$ added to initiate the reaction contained 1 μCi [γ-^{32}P]ATP. At 30-s intervals, 100-μl aliquots of the reaction medium were removed and added to 1.9 ml 8% TCA. Inorganic phosphate was then complexed with molybdate by the addition of 500 μl 3.75% ammonium molybdate in 3 N H_2SO_4 and extracted in 2.5 ml butyl acetate (16). The time course of release of P_i was linear over 3 min, and the slope of the best fit linear regression fit to the data was used in the calculation of initial rates of [γ-^{32}P]ATP hydrolysis.

Other Assays. Fluorescence quenching of 9-aminoacridine was assayed as described previously (5) in a medium identical to that used for proton influx measurements (see above). Excitation and emission wavelengths were 402 and 455 nm, respectively. Protein was determined by the method of Schaffner and Weissmann (17).

Source of Chemicals. ATP was obtained from Boehringer Mannheim as the disodium salt and used directly; [γ-^{32}P]ATP was obtained from Amersham (specific radioactivity 3 Ci/mmol). Butyl acetate was from Fisher and all other chemicals were from Sigma.

RESULTS

The addition of Mg:ATP resulted in alkalinization of the external medium of a membrane vesicle suspension (Fig. 2). The time course of alkalinization is similar to that of fluorescence quenching of 9-aminoacridine and levels off after about 6 min (Fig. 2). The leveling off of fluorescence quenching has been previously attributed to active H^+ influx and passive H^+ efflux approaching equilibrium (6). This equilibrium of fluxes is also apparent in measurement of the alkalinization of the vesicle

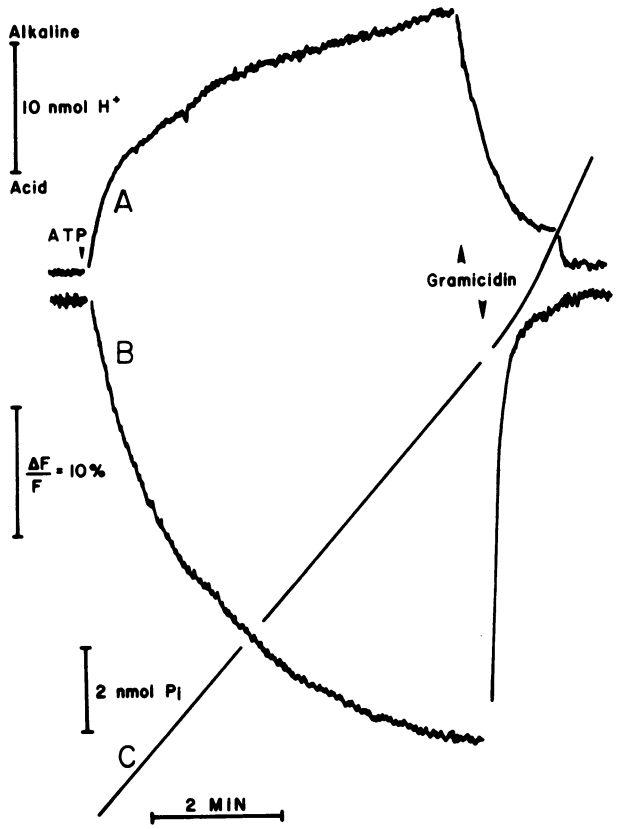


FIG. 2. Time course of ATP-dependent H^+ loss from the vesicle external medium (A), ATP-dependent quenching of 9-aminoacridine fluorescence (B), and ATP hydrolysis (C). All assays were performed as in "Materials and Methods."

exterior (Fig. 2). After the development of a pH gradient across the vesicle membrane, both the change in external medium pH and quenching of 9-aminoacridine fluorescence is completely reversed by addition of the channel-forming ionophore, gramicidin (Fig. 2). This complete reversal of the pH change indicated that the alkalinization resulted from H^+ transport into the membrane vesicles and not from the production of OH^- equivalents during ATP hydrolysis. At pH values as little as 0.2 unit below or above pH 6.13, production of H^+ or OH^- during ATP hydrolysis was sufficient to obliterate measurable pH changes resulting from H^+ transport. A pH of 6.13 has been shown theoretically to be near the isoprotonic pH for ATP hydrolysis to ADP and P_i under conditions utilized in this study (2). Although the presence of ADPase or adenylate kinase activity can further influence the production of H^+ or OH^-, medium pH between 6.10 and 6.25 has been found to be suitable for measuring ATP-dependent H^+ transport in submitochondrial particles (21) and gastric microsomal vesicles (13, 14).

The time course of ATP hydrolysis is also shown in Figure 2. The tracing is taken from a spectrophotometric recording of NADH oxidation (A_{340}) in the NADH-coupled ATPase assay ("Materials and Methods"). ATP hydrolysis is linear over the time span measured here (approximately 6 min) but increases sharply upon the addition of gramicidin. Based on measurements of the rates of ATP hydrolysis before the addition of gramicidin and on the initial rates of proton influx measured over time intervals before alkalinization begins to level off (typically over the interval between 6 and 30 s after ATP addition), the ratio of H^+ transported per ATP hydrolyzed could be calculated.

A representative experiment is detailed in Table I where initial rates of H^+ influx and ATP hydrolysis are measured in the presence of KCl or KNO_3. With KCl present, rates of both H^+ influx and ATP hydrolysis were higher than in the presence of KNO_3, in agreement with our previous results indicating that NO_3^- is a selective inhibitor of this anion-sensitive H^+-ATPase (5, 10). Contaminating phosphatase activity which did not contribute to H^+ transport would result in an underestimate of the H^+/ATP stoichiometry. We chose to base our calculations of the H^+/ATP ratio on NO_3^--sensitive H^+ transport and ATPase activities in order to avoid this source of error. The results shown in Table I are consistent with the presence of contaminating phosphatase activity which tends to lower the observed H^+/ATP stoichiometry. The level of contamination varied from preparation to preparation resulting in calculated H^+ATP stoichiometries measured in the presence of KCl showing wide variations between 0.8 and 1.7 whereas the ΔNO_3^- H^+/ATP stoichiometry was consistent from preparation to preparation as shown in Table II. The mean stoichiometry of H^+/ATP coupling calculated from the values in Table II was 1.96 ± 0.14 (SD) and, if we assume that the true stoichiometry is an integer value, this suggests that the true H^+/ATP stoichiometry is 2.

DISCUSSION

The approach used here to arrive at an H^+/ATP stoichiometry of 2 for the red beet anion-sensitive H^+-ATPase is best described as a kinetic approach since it has relied on measurements of initial rates of H^+ influx and ATP hydrolysis. Errors in these measurements, such as H^+ leaks, would tend to raise the H^+/ATP stoichiometry to values above 2. Thus, the value reported here is conservative. A further and necessary test of this estimated stoichiometry is to determine if this value is energetically sufficient to account for the $\Delta\bar{\mu}_{H^+}$ values measured *in vivo*. This amounts to a thermodynamic test of the value arrived at by a kinetic approach.

Table I. *H^+ Influx and ATPase Activity Measured in the Presence of Either KCl or KNO_3*

H^+ influx and ATPase activity were assayed at pH 6.13 as described in "Materials and Methds."

Salt	H^+ Influx	ATPase Activity	H^+/ATP
	μmol H^+/mg·h	*μmol P_i/mg·h*	
KCl	13.86	9.83	1.41
KNO_3	4.09	4.92	0.83
ΔNO_3^-	9.77	4.91	1.99

Table II. *Stoichiometry of NO_3^--Sensitive Proton Uptake to NO_3^--Sensitive ATP Hydrolysis*

H^+ influx was measured at pH 6.13 as described in "Materials and Methods." ATPase activity was assayed in experiments 1 to 4 with the NADH coupled assay and in experiments 5 and 6 by monitoring the release of ^{32}P from [γ-^{32}P]ATP.

Experiment	NO_3^--Sensitive H^+ Influx	NO_3^--Sensitive ATP Hydrolysis	H^+/ATP
	μmol H^+/mg·h	*μmol P_i/mg·h*	
1	6.35	3.38	1.88
2	7.26	4.05	1.79
3	9.77	4.91	1.99
4	11.39	5.73	1.99
5	10.95	4.95	2.21
6	7.47	3.89	1.92

Referring to equation 1, we can see that the maximum $\Delta\bar{\mu}_{H^+}$ sustainable by an H^+-ATPase with an H^+/ATP stoichiometry of 1 is ΔG_{ATP}. In order to evaluate this in measurable terms, we need an estimate for ΔG_{ATP} in the plant cytoplasmic environment, which can be arrived at by considering that:

$$\Delta G_{ATP} = \Delta G^{\circ\prime}{}_{ATP} + RT \ln \frac{[ADP]\,[P_i]}{[ATP]} \qquad (2)$$

where $\Delta G^{\circ\prime}{}_{ATP}$ is the standard free energy of ATP hydrolysis, R is the gas constant (*e.g.*, 1.987 cal mol^{-1} K^{-1}), and T is absolute temperature (296 K). The standard free energy of ATP hydrolysis ($\Delta G^{\circ\prime}{}_{ATP}$) at pH 7.5 and 1.0 mM Mg^{2+} is approximately −9.5 kcal mol^{-1} (2). ADP/ATP ratios are relatively constant at 0.3 in plant cells (7, 9), cytoplasmic phosphate concentration has been estimated to be 1 to 5 mM (19), and ATP concentrations close to 1 mM (11). Using these values, a temperature of 23°C, and equation 2, we can calculate cytoplasmic ΔG_{ATP} to be in range between −10 and −11 kcal mol^{-1}.

The electrochemical potential gradient for H^+ ($\Delta\bar{\mu}_{H^+}$) in units of kcal mol^{-1} is described by:

$$\Delta\bar{\mu}_{H^+} = F\Delta\psi - 2.303RT\,\Delta pH \qquad (3)$$

where $\Delta\psi$ is the membrane potential in mv, F is the Faraday constant and ΔpH is the pH gradient across the membrane. Substituting equation 3 into equation 1 and rearranging we can arrive at an equation relating ΔG_{ATP} to measurable quantities of $\Delta\psi$ and ΔpH across a membrane:

$$\frac{1}{n}\Delta G_{ATP} \geq F\Delta\psi - 2.303RT\,\Delta pH. \qquad (4)$$

From equation 4, we can calculate the maximal pH gradients sustainable by an H^+-ATPase operating at thermodynamic equilibrium over a range of possible membrane potentials (Fig. 3). The three lines in Figure 3 represent the maximum $\Delta\bar{\mu}_{H^+}$ sustainable by a H^+-ATPase with H^+/ATP stoichiometries of either 1, 2, or 3 so that areas above each line are thermodynamically impossible, given their respective stoichiometries, and ΔG_{ATP} of

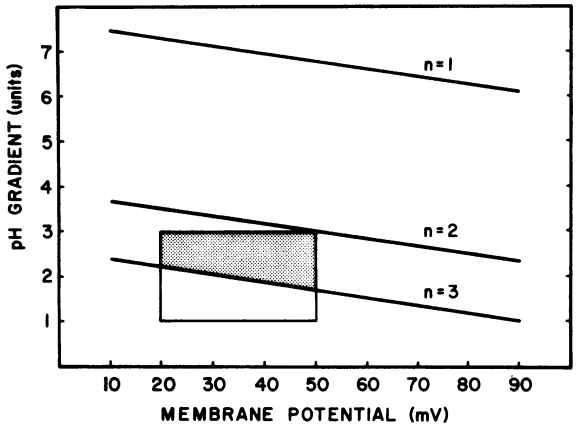


FIG. 3. Maximal pH gradients sustainable by a H^+-ATPase with H^+/ATP stoichiometries of 1, 2, or 3 over a range of membrane potentials. Curves were calculated from equation 4, assuming a ΔG_{ATP} of -10.5 kcal mol^{-1}.

-10.5 kcal mol^{-1} (equivalent to -450 mv mol^{-1}).

The box inset in Figure 3 represents the range of values for ΔpH and $\Delta\psi$ measured across the tonoplast *in vivo*. Values for ΔpH range from 1 to 3 units (15, 18) and values for $\Delta\psi$, from +20 to +50 mv (4, 20). Clearly, the entire range of physiological values could be accommodated by a H^+-ATPase with a H^+/ATP stoichiometry of either 1 or 2, since the physiological data fall below the respective lines. There is also a range of physiological data which could be accommodated by a H^+/ATP stoichiometry of 3. However, many of the physiological data fall above the $n = 3$ curve, and so are outside of the range of energetic competence for such a H^+-ATPase. This analysis suggests that the maximum H^+/ATP stoichiometry required to explain all of the physiological data is 2.

From the foregoing discussion it is apparent that a H^+/ATP stoichiometry of 2 is required to account for the higher range of $\Delta\bar{\mu}_{H^+}$ values measured across the tonoplast. However, only at the very highest reported values of ΔpH and $\Delta\psi$ (*e.g.*, $\Delta pH = 3$ units and $\Delta\psi = 50$ mv) does such a H^+-ATPase appear to be operating at thermodynamic equilibrium. The lower values of $\Delta\bar{\mu}_{H^+}$ may be explained in terms of a H^+-ATPase of variable stoichiometry which operates at thermodynamic equilibrium and is regulated only by energy supply. Small adjustments in $\Delta\bar{\mu}_{H^+}$ across the membrane would require that the H^+/ATP stoichiometry of such a variable H^+-ATPase assume noninteger values. Alternatively, and we feel more likely, the H^+-ATPase is of fixed H^+/ATP stoichiometry ($n = 2$) and does not operate at thermodynamic equilibrium but is under kinetic regulation by cellular factors not related, or only directly related, to energy supply. Such kinetic regulation would allow changes in $\Delta\bar{\mu}_{H^+}$ in response to cellular and environmental stimuli which are independent of energy supplied to the ATPase.

Studies of the energetics of malate accumulation in the vacuole of a Crassulacean acid metabolism plant, *Kalanchoë tubiflora*, allowed an estimate of the H^+/ATP stoichiometry of a tonoplast H^+-ATPase responsible for malic acid accumulation in this plant (19). The results of this study were based strictly on a thermodynamic consideration of the free energy available from ATP hydrolysis and $\Delta\bar{\mu}_{H^+}$ across the tonoplast *in vivo* and resulted in an estimate of an H^+/ATP stoichiometry of 2. The close agreement in values based on work *in vivo* and *in vitro* is not necessarily expected since the tonoplast H^+-ATPase is most likely under kinetic regulation *in vivo*. The fact that the two values do agree suggests that in Crassulacean acid metabolism plants the tonoplast H^+-ATPase does indeed operate near thermodynamic equilibrium at the end of the dark period when malate accumulation in the vacuole is greatest (19).

LITERATURE CITED

1. AL-AWQATI Q, TE DIXON 1982 Effect of electrochemical gradients on active H^+ transport in an epithelium. Curr Top Membr Transport 16: 163–174
2. ALBERTY RA 1968 Effect of pH and metal ion concentration on the equilibrium hydrolysis of adenosine triphosphate to adenosine diphosphate. J Biol Chem 243: 1337–1343
3. AUFFRET CA, DE HANKE 1981 Improved preparation and assay and some characteristics of Cl^--ATPase activity from *Limonium vulgare*. Biochim Biophys Acta 648: 186–191
4. BATES GW, MHM GOLDSMITH, TH GOLDSMITH 1982 Separation of tonoplast and plasma membrane potential and resistance in cells of oat coleoptiles. J Membr Biol 66: 15–23
5. BENNETT AB, SD O'NEILL, RM SPANSWICK H^+-ATPase activity from storage tissue of red beet. I. Identification and characterization of an anion-sensitive H^+-ATPase. Plant Physiol 74: 538–544
6. BENNETT AB, RM SPANSWICK 1983 Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles. Kinetic analysis of Cl^- effects on a proton-translocating ATPase. J Membr Biol 71: 95–107
7. BIELESKI RL 1973 Phosphate pools, phosphate transport, and phosphate availability. Annu Rev Plant Physiol 24: 225–252
8. DIXON JF, LE HOKIN 1980 The reconstituted (Na, K)-ATPase is electrogenic. J Biol Chem 255: 10681–10686
9. GRONEWOLD JW, JB HANSON 1982 Adenine nucleotide content of corn roots as affected by injury and subsequent washing. Plant Physiol 69: 1252–1256
10. O'NEILL SD, AB BENNETT, RM SPANSWICK 1983 Characterization of a NO_3^--sensitive H^+-ATPase from corn roots. Plant Physiol 72: 837–846
11. PETRAGLIA T, RJ POOLE 1980 ATP levels and their effects on plasmalemma influxes of potassium chloride in red beet. Plant Physiol 65: 969–972
12. POOLE RJ 1978 Energy coupling for membrane transport. Annu Rev Plant Physiol 29: 437–460
13. RABON EC, TL MCFALL, G SACHS 1982 The gastric [H, K]-ATPase: H^+/ATP stoichiometry. J Biol Chem 257: 6296–6299
14. REENSTRA WW, JG FORTE 1981 H^+/ATP stoichiometry for the gastric ($K^+ + H^+$)-ATPase. J Membr Biol 61: 55–60
15. ROBERTS JKM, PM RAY, N WADE-JARDETZKY 1980 Estimation of cytoplasmic and vacuolar pH in higher plant cells by ^{31}P-NMR. Nature (Lond) 283: 870–872
16. SANUI H 1974 Measurement of inorganic orthophosphate in biological materials: Extraction properties of butyl acetate. Anal Biochem 60: 489–504
17. SCHAFFNER W, C WEISSMANN 1973 A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal Biochem 56: 502–514
18. SMITH FA, JA RAVEN 1979 Intracellular pH and its regulation. Annu Rev Plant Physiol 30: 289–311
19. SMITH JAC, G MARIGO, U LÜTTGE, E BALL 1982 Adenine-nucleotide levels during Crassulacean acid metabolism and the energetics of malate accumulation in *Kalanchoë tubiflora*. Plant Sci Lett 26: 13–21
20. SPANSWICK RM, EJ WILLIAMS 1964 Electrical potentials and Na, K, and Cl concentrations in the vacuole and cytoplasm of *Nitella translucens*. J Exp Bot 15: 422–427
21. THAYER WS, PC HINKLE 1973 Stoichiometry of adenosine triphosphate driven proton translocation in bovine heart submitochondrial particles. J Biol Chem 248: 5395–5402
22. WALKER NA, FA SMITH 1977 The H^+-ATPase of the *Chara* cell membrane: its role in determining membrane P.D. and cytoplasmic pH. *In* M Thellier, A Monnier, M DeMarty, J Dainty, eds, Transmembrane Ionic Exchanges, Centre National de la Recherche Scientifique, Paris, pp 258–261