Effect of Temperature on the Plasma Membrane and Tonoplast ATPases of Barley Roots

Comparison of Results Obtained with Acridine Orange and Quinacrine

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ABSTRACT

The effect of temperature on the rate of proton transport and ATP hydrolysis by plasma membrane (PM) and tonoplast (TN) ATPases from barley (Hordeum vulgare L. cv CM 72) roots were compared. Rates of proton transport were estimated using the fluorescent amine dyes quinacrine and acridine orange. The ratio between rate of transport and ATP hydrolysis was found to depend on the dye, the temperature, and the type of membrane. The PM ATPase had an estimated Arrhenius energy of activation (Eₐ) of approximately 18 kilocalories per mole for ATP hydrolysis, and the Eₐ for proton transport was best estimated with acridine orange, which gave an Eₐ of 18 kilocalories per mole. The TN ATPase had an Eₐ for ATP hydrolysis of approximately 18 kilocalories per mole and the Eₐ for proton transport was best estimated with quinacrine, which gave an Eₐ of 10 kilocalories per mole. Acridine orange did not give an accurate estimate of Eₐ for the TN ATPase, nor did quinacrine for the PM ATPase. Reasons for the differences are discussed. Because it was suggested (AJ Pope, RA Leigh [1988] Plant Physiol 86: 1315–1322) that acridine orange interacts with anions to dissipate the pH gradient in TN vesicles, the complex effects of NO₃⁻ on the TN ATPase were also examined using acridine orange and quinacrine and membranes from oats and barley. Fluorescent amine dyes can be used to evaluate the effects of ions, substrates, inhibitors, and temperature on transport but caution is required in using rates of quench to make quantitative estimates of proton fluxes.

Various membranes including the plasma membrane (PM) and tonoplast (TN) have been hypothesized to be primary sites of chilling injury because chilling temperatures may have direct effects on the fluidity, viscosity, or phase-state of membrane lipids (13, 23, 26 and references therein). Ion leakages caused by the effect of temperature on membrane lipids or proteins have been cited as potential causes of chilling injury or freezing damage (13, 28) and the process of cold acclimation may include modification of lipid and protein components of the PM or TN (13, 21, 28). One method to test hypotheses about the direct effect of temperature on the TN and PM is to examine the effects of temperature on isolated vesicles (11). Analysis of the effect of temperature on isolated PM and TN vesicles from plant tissues also can provide information on the energy requirements of ion transport, the mechanisms of action of membrane transport systems (16), and the responses of plants to stressful high temperatures. Since barley has been used as an example of a plant that does not undergo chilling injury (26), it was of some interest to examine the effects of temperature on transport by barley membranes.

In order to measure the effect of variables such as temperature and ions on the TN and PM ATPase, it is important to find methods to measure transport that are not affected significantly by the conditions being tested. If temperature- and ion-insensitive methods are not available, then the effects of temperature and ions on the techniques that are used must be defined. Fluorescent dyes such as acridine orange and quinacrine are commonly used in procedures for estimating rates of proton transport by membrane vesicles. However, Pope and Leigh (25) discussed various artifacts that occurred when acridine orange was used to measure pH gradients formed in TN vesicles obtained from red beet storage tissue, and Grzesiek and Dencher (15) described the artifacts that can occur when 9-aminoacridine is used to measure pH gradients. In this paper, the effects of temperature on ATP hydrolysis and on proton transport by the TN and PM ATPases of barley roots are described. During the course of the experiments it became clear that estimates of the effect of temperature on transport rates obtained with acridine orange or quinacrine could be quite different, and that the same dye might not be the best choice for the PM as for the TN. Some limitations on the use of quinacrine and acridine orange are described with respect to the two different membranes.

MATERIALS AND METHODS

Plant Material

Seeds of barley (Hordeum vulgare L. cv CM 72) and oats (Avena sativa L. var Lang) were grown for 7 d at 22°C above an aerated solution containing 0.1 mM CaSO₄ (10).
Membrane Preparations

TN and PM vesicles were prepared from barley roots as previously described (12). TN vesicles were collected from the 22/30% interface and PM vesicles from the 34/40% interface of a sucrose step gradient. The membranes were washed with a buffered solution of 150 mM KCl, resuspended in a suspension buffer consisting of 0.25 mM sucrose and 1 mM DTT in 5 mM Pipes-KOH (pH 7.2), and stored at -70°C prior to use. There was little cross-contamination between the PM and TN membranes as judged by enzyme assays, protein patterns on SDS gels, and immunoblots using antibodies to PM and TN proteins (12). Both the PM and TN fractions contained sufficient amounts of sealed vesicles with the correct sidedness to measure transport by the respective H+-ATPases, so that the transport properties of the two membranes could be compared in vitro.

TN enriched membrane fractions were prepared from oats by the method described by Schumaker and Sze (27) and by modifications of that method. The homogenization buffer consisted of 0.25 mM mannitol, 2 mM EDTA, 1 mM DTT, and 0.1% BSA in 25 mM Hepes BTP (pH 7.5) (27) or was a modified version of the homogenization buffer used for barley. Other modifications are indicated in the text. A 10,000 to 100,000g pellet was resuspended in suspension buffer consisting of 0.25 mM mannitol, 1 mM DTT and 5 mM Hepes BTP (pH 7.5), and layered over 10 mL of 10% dextran in the same buffer. Following centrifugation for 2 h at 80,000g the TN-enriched fraction was collected from the sample/dextran interface and stored in 1 mL aliquots at -70°C until assayed.

Proton Transport Assays

The standard assay solution for proton transport consisted of 0.25 mM sucrose in 5 mM Pipes KOH, at pH 6.5 to 6.8 for the PM and pH 7.5 for the TN ATPase, plus 2 mM acridine orange or 5 mM quinacrine. Mannitol was used instead of sucrose for assays of oat membranes. The concentrations of ATP, MgCl2, salts, and inhibitors that were used are indicated in the figure legends. Previous experiments (9, 12) showed that proton transport by the TN ATPase was completely inhibited by a combination of 100 mM KNO3 and 1 mM valinomycin, and the pH optimum was between 7.5 and 8.0. Previous experiments (12; and our unpublished data) showed that proton transport by the PM ATPase was stimulated by valinomycin and KNO3, the pH optimum was between pH 6.5 and 7.0, and transport was completely inhibited by 100 μM DCCD. The initial rate of transport was inhibited 80% by 500 μM valinomycin. Single vials of frozen membranes were thawed, stored on ice, and then used for assays for up to 3 h without a significant change in rates.

Proton transport was assayed as the quenching of acridine orange or quinacrine fluorescence using a Farrand3 System 3 spectrofluorometer. Fluorescence was measured with an excitation wavelength of 495 nm and an emission wavelength of 525 nm for acridine orange, and an excitation wavelength of 426 nm and emission wavelength of 495 nm for quinacrine.

Temperatures from 0 to 45°C were maintained with a flow of ethylene glycol-based antifreeze through a water-jacketed cuvette holder and connected to a Neslab water bath. The temperature of each assay was determined by inserting a thermocouple into the cuvette at the end of the assay. Temperatures accurate to 0.1°C were measured using a Fluke digital thermometer. Data were collected and analyzed by using Asystant+ software (ASYST Software Technologies) and an IBM compatible PC-XT. To determine initial rates, the first 30 or 60 s of data for the quench curve were selected and the slope determined by linear regression, with regression coefficients >0.95. When quinacrine was used to assay transport by the PM ATPase, there was a lag between the addition of ATP and the beginning of ATP-dependent quench, especially at low temperatures. In that case the data for 30 s of the steepest portion of the quench curve were used to approximate the initial rate. Data shown are for representative experiments.

ATPase Assays

ATP hydrolysis was assayed in the same assay solutions as were used for proton transport, minus the fluorescent dye, and total Pi released after 20 min was determined. ATP hydrolysis by the PM fraction was assayed at pH 6.8 in the presence of KNO3 and valinomycin and in the presence or absence of 100 μM DCCD. The difference is referred to as the DCCD-inhibited ATPase activity. In the PM fraction 80 to 90% of the total ATPase activity was stimulated by Mg2+, unaffected by azide, oligomycin or molybdate, and inhibited by 100 μM DCCD (12). ATP hydrolysis was inhibited 50% by 200 to 500 μM vanadate. ATP hydrolysis by the TN fraction was assayed in the presence of 50 mM KCl or 50 mM KNO3 and the difference referred to as the NO3−-inhibited ATPase. Less than 50% of the activity was inhibited by NO3− and the remainder of the activity was stimulated equally by Ca2+ or Mg2+ and was not specific for ATP (9, 10). Inorganic Pi was determined by a modification of the method of Lebel (1, 17). Vanadate interfered with the Pi determination, so this interference was corrected for when vanadate was used in ATPase assays. ATP hydrolysis was linear with time for 30 min at temperatures up to 36°C, the highest temperature tested. Data shown are for representative experiments.

Arrhenius Energy of Activation

The Ea for ATP hydrolysis and for quench response was calculated using the equation

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Ea = \frac{T_2 T_1}{T_2 - T_1} \cdot 4.576 \left( \log k_2 - \log k_1 \right)
\]

where \( T \) is temperature (°K) and \( k \) is the rate of reaction. The Ea values given in the text are calculated from the representative experiments shown in the figures. Similar values were calculated in the replicate experiments.

3 Mention of a specific product name by the U.S. Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.
RESULTS

Effect of Temperature on the PM ATPase from Barley Roots

The effect of temperature on the Mg\(^{2+}\)-requiring, DCCD-inhibited, NO\(^{-}\)-insensitive ATPase was compared with the effect of temperature on the NO\(^{-}\}-insensitive proton transport in the PM fraction. ATP hydrolysis was assayed in the presence or absence of 100 μM DCCD and the DCCD-inhibited component of the ATPase was taken as an estimate of activity of the proton translocating ATPase. Vanadate-sensitive ATPase was not used, because the effect of vanadate on transport by sealed PM vesicles was complex (4, 12) and vanadate did not give complete inhibition of proton transport. ATP hydrolysis by the PM ATPase from barley roots was assayed as a function of temperature from 0 to 45°C (Fig. 1). The rate of ATP hydrolysis increased with temperature and reached a maximum around 40°C (Fig. 1A). Arrhenius plots of the data (Fig. 1B) indicate that the log of the rate for ATP hydrolysis increased in a linear fashion as a function of temperature between 4 and 40°C. The \(E_a\) for ATP hydrolysis was 18 kcal/mol for the DCCD-inhibitable ATPase.

Acridine orange and quinacrine were used to estimate relative rates of proton transport by the PM ATPase as a function of temperature (Figs. 2 and 3). Several difficulties were encountered in making the measurements, particularly with quinacrine. The amount of quench per mg protein was less with quinacrine than with acridine orange, and the fluorescence intensity of 5 μM quinacrine was less than that of 2 μM acridine orange. The resulting high signal-to-noise ratio for quinacrine (Fig. 2, B and C) made it more difficult to measure the low rates of transport that were obtained at temperatures below 20°C. Unless large amounts of protein were used, it was difficult to distinguish ATP-dependent quench of quinacrine fluorescence from background noise in the recordings. At temperatures above 20°C, rates were sufficiently high that small amounts of protein could be used for the measurements and a single membrane preparation could be used for many assays. Upon addition of NH\(_4\)Cl to the vesicles, to collapse the ΔpH, fluorescence of acridine orange returned to the original level but fluorescence of quinacrine did not (Fig. 2). Measurement of the initial rate of quench was also difficult with quinacrine because the response of the dye to formation of a ΔpH was delayed (Fig. 2B). After adding ATP, there was typically a lag before the fluorescence of quinacrine began to decrease; the lag probably occurred while sufficient quinacrine entered the vesicles to initiate self-quenching by the concentrated dye molecules. There was a small lag with acridine orange below 20°C (Fig. 2A).

Using either dye, the initial rate of quench increased greatly from 10 to 30°C and reached a maximum near 40°C (Fig. 3). The \(E_a\) for proton transport was 19.1 kcal/mol for the data from 13 to 37°C when acridine orange was used (Fig. 3B). This was similar to the \(E_a\) of 18 kcal/mol for DCCD-inhibited ATP hydrolysis. The calculated \(E_a\) for quinacrine was much higher, being 30 kcal/mol for the data from 22 to 40°C (Fig. 3B). One reason for a high value for the \(E_a\) may be the fact that the lag in the quench response increased as the temperature decreased, so that initial rates of transport at low temperatures were underestimated.

Effect of Temperature on the TN ATPase from Barley Roots

ATP hydrolysis by the TN membranes increased as a
function of temperature form 0 to 40°C and reached a maximum around 40°C (Fig. 4A). Arrhenius plots of the data indicated that the log of the rate of ATP hydrolysis was approximately linear over the range of temperatures used (Fig. 4B) and the Ea for ATP hydrolysis in the presence of KCl or KNO_3 as well as the NO_3^- -inhibited ATPase were all between 10 and 12 kcal/mol. The NO_3^- -inhibitable component of ATP hydrolysis was assumed to represent the activity of the TN ATPase (9).

The effect of temperature on proton transport by the TN ATPase also was measured (Fig. 5). When acridine orange was used to estimate initial rates of transport, the effect of temperature on transport was very different from the effect of temperature on ATP hydrolysis. Relatively high rates of quench were measured at low temperatures, from 2 to 15°C (Fig. 5A), but the rate declined above 15 or 20°C (Fig. 5A) and was very low above 30°C. The results obtained with quinacrine were more similar to those for ATP hydrolysis (Fig. 5A). The initial rate of quench increased from 9 to 38°C and decreased above 40°C and the Arrhenius plot of the data (Fig. 5B) shows a linear increase in log of the initial rate of transport from 9 to 38°C. The Ea for transport of 9.7 kcal/mol that was obtained with quinacrine was similar to the Ea of 10.3 kcal/mol for the NO_3^- -inhibited ATPase (Fig. 4B).

The problem of an initial lag with quinacrine was not so pronounced as with the PM ATPase. One reason may be that the TN ATPase was assayed at pH 7.5, creating a small initial pH gradient prior to the addition of ATP.

**Ratios of Transport to ATP Hydrolysis**

Initial rates of proton transport were expected to remain proportional to initial rates of ATP hydrolysis when the rates were altered by varying the assay conditions (2). A comparison of Figures 1 to 5 shows that sometimes this was not true when fluorescence quench techniques were used to estimate transport rates. To facilitate the comparison, the ratio of rate of quench to rate of ATP hydrolysis was calculated for the PM and TN ATPases for both quinacrine and acridine orange at a number of temperatures (Table I). The DCCD-inhibited ATPase was used to estimate the rate of ATP hydrolysis by the PM ATPase, and the NO_3^- -inhibited ATPase was used to estimate the rate of ATP hydrolysis by the TN ATPase. Some variability was introduced into the calculations as a result of calculating the DCCD-inhibited ATPase or the NO_3^- -inhibited ATPase and then dividing that number into percent quench to obtain the ratio.

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**Figure 2.** Effect of temperature on the time course for ATP-dependent quench of fluorescence by the PM ATPase. Transport was assayed as in Figure 1 with 5 mM Pipes-KOH (pH 6.8). Transport was initiated by the addition of 3 mM Tris ATP and halted by the addition of 3 mM NH_4Cl (AM). A, transport was assayed with 2 μM acridine orange, using 28 μg protein per assay. Traces are normalized so that the quench curves begin at the same fluorescence intensity. B and C, Transport was assayed with 5 μM quinacrine, using 35 μg protein per assay (B), or 28 μg protein per assay (C). Numbers indicate assay temperature (°C). Origin of traces is offset for clarity. Membranes used in Figure 2A were from roots grown with 100 mM NaCl.
The ratio of transport to ATP hydrolysis for the PM ATPase did not vary much as a function of temperature when acridine orange was used and ranged from 0.3 to 0.4%/nmol ATP at 12 to 37°C. Quinacrine gave a lower ratio of quench to ATPase, and the ratio increased greatly from 20 to 40°C, probably because the initial rates of protein transport were underestimated at lower temperatures, as discussed above.

The TN ATPase gave a ratio of quench to ATPase with quinacrine of 1.6 to 2.7%/nmol ATP for temperatures between 12 and 37°C. When acridine orange was used the highest ratio of quench to ATPase was 9.8%/nmol ATP at 12°C, but the ratios declined at higher temperatures. In order to decrease the variability in the calculations, the ratios for the TN ATPase were also calculated by assaying total Pi released during a 10 min fluorescence quench assay (Fig. 6). For quinacrine, ratios of quench to total Pi were approximately 0.5%/nmol and declined slightly as temperatures were...
Effect of Temperature on Steady State pH

The effect of temperature on the steady state quench of fluorescence was also estimated. The steady state ΔpH is achieved when proton influx via the pump equals proton efflux. The steady state quench that was attained at each temperature for the PM and TN ATPases is shown in Figure 7. The steady state quench obtained with acridine orange was as much as 10 times greater than that obtained with quinacrine for the PM ATPase (Fig. 7A). Steady state quench for

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\text{Figure 5. A, Initial rates of proton transport by the TN ATPase as a function of temperature. Transport was assayed with } 1 \text{ mM Tris-ATP, } 1 \text{ mM MgCl}_2, 50 \text{ mM KCl, } 1 \text{ mM EGTA, and } 5 \text{ mM Pipes KOH at pH 7.2 with } 2 \mu\text{M acridine orange (O) using } 10 \mu\text{g protein per assay or at pH 7.5, with } 5 \mu\text{M quinacrine (M) and } 48 \mu\text{g protein per assay. B, Arrhenius plot of the data in A. The line was fitted by linear regression analysis of the data from 10 to } 37^\circ\text{C for quinacrine (Ea } = 9.8 \text{ kcal/mol, } r = -0.9276).}
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raised from 16 to 40°C. For acridine orange, ratios of quench to total Pi declined steeply as a function of temperature, ranging from 3%/nmol at 16°C to 0.5%/nmol at 40°C.

**Effect of Temperature on Steady State pH**

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*Acridine orange. **Quinacrine. aDCCD-inhibited ATPase from Figure 1. bFrom Figure 3. *Not determined. cND* inhibited ATPase from Figure 4. dFrom Figure 5.
quenched by acridine may be underestimated because there was not complete recovery of quench upon addition of NH₄Cl (Fig. 2, B and C). The greatest values for steady state quench were attained near 20°C for acridine orange, but between 30 to 40°C for quinacrine. The steady state quench also was greater with acridine orange than with quinacrine for the TN ATPase (Fig. 7B). It reached a maximum from 10 to 20°C for acridine orange and from 15 to 30°C with quinacrine. At low temperatures, the steady state quench attained by the TN ATPase with acridine orange was very large, as much as 8.5%/µg protein at 2°C and 10%/µg at 10°C. If it is assumed that the steady state quench is a measure of the steady state ΔpH, then for both the PM and TN ATPases there was a range of temperatures where the steady state ΔpH was insensitive to temperature. In the presence of acridine orange the values for the ΔpH formed by the PM ATPase were of similar magnitude from 10 to 30°C, whereas in the presence of quinacrine the values for the ΔpH were approximately the same from 20 to 40°C. The values for the ΔpH formed by the TN ATPase were approximately the same, regardless of temperature, from 5 to 20°C for acridine orange or from 15 to 35°C for quinacrine, then declined at higher temperatures.

**Effect of Preincubation at Higher Temperatures**

The decline in initial rate of quench and steady state quench of acridine orange that was observed for the TN ATPase at temperatures above 20°C could be due to an irreversible process, such as degradation of the physical structure of the membrane by lipases or proteases. To test whether the decline was reversible or irreversible, the TN membranes were preincubated at higher temperatures before being assayed at 11°C (Fig. 8). When the membranes were preincubated at 24 or 32°C for 10 min and then assayed at 11°C, the rates were similar to the rates at 11°C for untreated membranes. The absence of an effect of pretreatment at higher temperatures, together with the very different temperature profile obtained using quinacrine, argues against physical damage to the TN membranes during short incubation periods at temperatures as high as 32°C. Another explanation for the decline in rates

![Figure 7. Steady state quench of fluorescence as a function of temperature. Transport was initiated by addition of ATP and fluorescence intensity was measured long enough to reach a steady state with no change in fluorescence intensity with time (or for 30 min). The steady state quench was calculated as the % fluorescence recovered upon addition of NH₄Cl. A, Steady state quench for the PM ATPase, measured with acridine orange (○) or quinacrine (■); B, steady state quench for the TN ATPase, measured with acridine orange (○) or quinacrine (■).](image)

![Figure 8. Effect of preincubation at different temperatures upon steady state quench by the TN ATPase. Transport was assayed with 2 μM acridine orange, 50 mM choline Cl, 1 mM MgCl₂, 2 mM EGTA, and 5 mM Bis-Tris-Propane/Mes (pH 7.7), using 25 μg protein per assay. Vesicles were held on ice, then (a) assayed at 11°C; (b) preincubated 10 min at 24°C, returned to the ice, then assayed at 11°C; (c) preincubated 10 min at 32°C, returned to the ice, then assayed at 11°C; or (d) assayed at 32°C. Transport was initiated by the addition of 1 mM ATP and halted by the addition of 3 mM NH₄Cl (AM).](image)
observed with acridine orange above 20°C might be that it directly inhibited the TN ATPase. However, neither 2 μM acridine orange nor quinacrine had any effect on the rate of ATP hydrolysis (not shown). The data are consistent with the hypotheses of Pope and Leigh (25) that acridine orange within the vesicles combines with anions such as Cl\(^-\) or NO\(_3\)^- to form lipophilic ion pairs that dissipate the pH gradient, and that this interaction is temperature dependent.

**Effect of NO\(_3^-\) on the Transport Assays**

Because various published results from this laboratory were obtained by using acridine orange to measure transport by the TN ATPase, it was important to determine if the results were reproducible with quinacrine. Similar measurements of Na\(^+/\)H\(^+\) exchange (14) and of collapse of the ΔpH upon addition of valinomycin (9, 12) were obtained with quinacrine or acridine orange (J Garbarino, FM DuPont, unpublished data) and it was easier to measure the Na\(^+/\)H\(^+\) exchange with acridine orange because of the greater sensitivity of the dye to ΔpH. There was also a question about measurements which demonstrated that the effects of NO\(_3^-\) on proton transport by the TN ATPase varied as a function of temperature (9). At low temperatures, NO\(_3^-\) did not immediately inhibit proton transport as measured by quench of acridine orange fluorescence. Since Pope and Leigh (25) reported that NO\(_3^-\) interacted with acridine orange but not quinacrine in a temperature dependent manner, it was possible that the two-phase time course that was observed in the presence of NO\(_3^-\) (9) was an artifact associated with the use of acridine orange. Therefore, the effects of NO\(_3^-\) on the TN ATPase were examined using quinacrine (Fig. 9). In the presence of KNO\(_3\), a two-phase time course of fluorescence was obtained for quinacrine similar to that previously observed with acridine orange. Addition of ATP initiated quench of fluorescence in the presence of 50 mM KNO\(_3\), indicating that an ATP-dependent pH gradient was formed and only subsequently dissipated. There was a lag of 1.5 min before any inhibitory effect of NO\(_3^-\) was observed. Quinacrine plus NO\(_3^-\) gave a greater extent of quench than was obtained with acridine orange plus NO\(_3^-\) (not shown) as might be predicted if the pH gradient was dissipated more rapidly in the presence of acridine orange than in the presence of quinacrine (25).

It frequently has been reported that high concentrations of NO\(_3^-\) completely inhibit transport by the TN ATPase (25 and references therein). Therefore, a test was made to determine if the variable effects of NO\(_3^-\) were unique to TN from barley roots. A TN enriched membrane fraction was prepared from oat roots by the method of Schumaker and Sze (27) and the effects of KNO\(_3\) on transport were determined. No proton transport was detected in the presence of 50 mM KNO\(_3\) (not shown). However, when oat roots were homogenized in a similar buffer to that used for the barley roots, a two-phase time course for ATP-dependent proton transport was observed (Fig. 10). The main differences between the two buffers were that the former (27) contained BSA and was adjusted to pH 7.5 and that the procedure used a smaller volume of homogenization buffer per gram fresh weight of roots, whereas the latter (Fig. 10) had a four-fold higher concentration of EDTA, a fourfold higher concentration of DTT, and was adjusted to pH 8.0. The effects of each of these variables were tested separately. Addition of ATP caused a pH gradient to form in the presence of NO\(_3^-\) when the membranes were prepared using the higher pH buffer or buffer with 8 mM EDTA. Changing the concentration of DTT did not affect the results, and adding BSA to the barley root buffer merely gave a higher estimate of protein concentration for the membrane preparation.

**DISCUSSION**

Several methods are commonly used to measure ΔpH in membrane vesicles in order to measure transport by H\(^+\)-ATPases in vitro (29 and references therein). A convenient method to estimate rates of transport is to measure the initial rate of quench of a fluorescent amine dye such as quinacrine or acridine orange (2). The assumption made is that the initial rate of quench is proportional to the initial rate of formation of the ΔpH, which is proportional to the proton influx. The results in this paper demonstrate that this assumption is not always correct.

Estimates of the effect of temperature on rates of transport by two barley ATPases depended on whether acridine orange or quinacrine was used and on which ATPase was measured. For the PM ATPase, the rate of quench was nearly proportional to the rate of ATP hydrolysis from 12 to 37°C if acridine orange was used. When quinacrine was used, however, there was a pronounced lag at the start of the quench curve which made it difficult to estimate initial rates. The ratio of quench-
EFFECTS OF TEMPERATURE ON TWO H⁺-ATPases

Figure 10. A comparison of the effect of KNO₃ and KCl on proton transport by a TN-enriched membrane fraction from oat roots. Transport was assayed with 2 μM acridine orange and 82 μg protein at 20°C, with 50 mM KCl or 50 mM KNO₃. ATP and NH₄Cl (AM) were added as indicated. Membranes were prepared using a homogenization buffer similar to that used for the barley roots.

to-ATPase for quinacrine increased with temperature and the estimated Ea for transport was nearly twice that for ATP hydrolysis by the PM ATPase. For the TN ATPase, the rate of quench was proportional to the rate of ATP hydrolysis from 10 to 37°C when quinacrine was used. It was not as difficult to measure transport with quinacrine at low temperatures for the TN ATPase. The problem of an initial lag with quinacrine was not serious and the TN ATPase had a lower Ea than the PM ATPase, so the rates did not decrease as much when the temperature was lowered. When acridine orange was used for the TN ATPase, however, there was little correspondence between the effects of temperature on transport and on ATP hydrolysis. It should be noted that similar high rates of quench of acridine orange were not observed at low temperatures for TN vesicles from tomato cells (11). Transport by the tomato TN H⁺-ATPase increased from 5 to 30°C with an Ea of approximately 10 kcal/mol.

Acridine orange was a more sensitive indicator of the ΔpH for both PM and TN and a larger percentage of acridine orange than quinacrine was taken up in response to the same size ΔpH. In order to understand the differences between the interactions of the two dyes with the PM and TN, it would help to understand the mechanism of pH-dependent quench, but this is not known. One mechanism of quench may be dye-dye interactions that occur because the molecules are concentrated within the vesicles. Another may be binding of dye to the membranes. If the dye behaves like a permanent weak base the distribution of the dye between the interior of the vesicles and the external medium in response to a ΔpH is described by the following equation:

\[ \text{pH}_\text{e} - \text{pH}_\text{i} = \log(A_i/A_o) + \log(V_o/V_i) \]

where \( A \) is the total amount of dye and \( V \) is the volume (18). For this ideal behavior, a plot of \( \log(A_i/A_o) \) versus ΔpH gives a straight line with a slope of 1 and the y-intercept equals \( \log(V_o/V_i) \). Over a certain range of ΔpH, acridine orange has been reported to give a linear plot for \( A_i/A_o \) (2, 18, 24) but the slope is not equal to 1, and more dye is accumulated than is predicted. It is speculated that the dye also binds to negative sites on the membrane (18). If that is the case, then both the bound and free dye accumulate in response to ΔpH and are released by addition of ionophores.

It is possible to estimate the actual concentration of dye that is accumulated within the vesicles. Estimates for the internal volume of similar vesicles ranged from 0.5 to 10 μL/mg protein (20, 24). A preliminary estimate for the volume of the barley TN vesicles is 2 μL/mg protein (FM DuPont, JJ Windle, unpublished data). Given a maximum steady state quench of 1%/μg protein (TN, Fig. 7), measured in 3 mL of 2 μM acridine orange, and a volume of 2 μL/mg protein, then a concentration of at least 30 mM acridine orange was achieved in the TN vesicles, which is 15,000 times the external concentration. Similarly, the maximum steady state quench was 0.7%/μg protein (TN, Fig. 7) in 5 μM quinacrine, giving an internal concentration of 53 mM quinacrine, 10,000 times the external concentration. In the same vesicles there was only a 15-fold concentration gradient for methylamine (FM DuPont, DC Bush, unpublished data). Pope and Leigh (25) suggested that acridine orange and anions interact within TN vesicles and liposomes and somehow dissipate a performed ΔpH. The high concentrations of acridine orange that are accumulated in the TN vesicles may contribute to this tendency to dissipate the ΔpH, but are not sufficient to explain it, since even higher concentrations of dye were achieved with quinacrine.

It is difficult to determine the effects of anions on the TN ATPase if anions also interact with the dye that is used to measure proton transport. The results in this paper demonstrate that the degree to which NO₃⁻ inhibits the TN ATPase also depends on the method that is used to prepare the membranes. NO₃⁻ prevented the formation of a ΔpH when the TN vesicles were prepared by one method, but the TN ATPase was much less sensitive to NO₃⁻ when the vesicles were prepared by a different method. One hypothesis to explain this variable sensitivity to NO₃⁻ is that inhibition by NO₃⁻ is a sign of damage to the ATPase, whereas the enzyme might be relatively insensitive to NO₃⁻ in vivo.

There were no serious difficulties in using acridine orange together with NO₃⁻ to measure the ΔpH formed by the PM ATPase (9, 12). The PM ATPase maintained a ΔpH in the presence of acridine orange and NO₃⁻ for more than 30 min. The differences between the effect of acridine orange on PM vesicles, TN vesicles from barley, TN vesicles from tomato (11) and liposomes (25) might be caused by differences in lipid or protein composition. The barley PM fraction had a higher ratio of sterols to phospholipids than the TN-enriched...
fraction, whereas the latter was enriched in sphingolipids (D Brown, FM DuPont, unpublished data). The interaction of the dye with the membrane may be affected by differences in charge density on the outer surface of the vesicles because of differences in lipid head group composition, exposed portions of membrane proteins, or degree of glycosylation. Another possibility is that acridine orange interacts with a transport protein in the barley TN.

The estimates of the transport-to-ATPase ratio for the TN and PM ATPases permit a crude comparison of the proportion of the ATPases that were in sealed vesicles with the orientation needed to generate a ΔpH (right-side out TN and inside-out PM). The transport-to-ATPase ratio for the TN ATPase was much higher than the ratio for the PM ATPase (Table I). With quinacrine for example, the ratio of quench to ATPase for the TN ATPase was at least 10 times higher than for the PM ATPase. This suggests that there were many more sealed TN vesicles than sealed PM vesicles. Other factors may also contribute to the differences in ratios. A difference in stoichiometry of H⁺ transported per ATP hydrolyzed could make a twofold difference since the estimated stoichiometry for the fungal PM ATPase was one H⁺/ATP (24) whereas the estimate for the TN ATPase from red beet storage tissue was two H⁺/ATP (3). If the internal buffering capacity of the PM vesicles was larger than that of the TN vesicles, then the ratio of ATPase to quench would be lower for the PM than the TN vesicles. Additional factors which might contribute to the difference could be a DCCD-inhibited ATPase which is different from the PM H⁺-ATPase, and a tendency for the TN vesicles to accumulate more molecules of amine dye than the PM vesicles per unit of ΔpH.

There were no obvious deviations from linearity for the Arrhenius plots of the data for ATP hydrolysis by the TN ATPase from 4 to 40°C. The Ea was approximately 10 kcal/mol for ATP hydrolysis and also for proton transport measured using quinacrine over the range of 10 to 37°C. The drop off in rates of quench of quinacrine at temperatures below 10°C may reflect problems in using quinacrine at low temperatures. The Arrhenius plots for the PM ATPase were fitted by a straight line from 4 to 38°C for transport measured with acridine orange and from 4 to 40°C for ATP hydrolysis. Caldwell (6) and Caldwell and Whitman (8) analyzed the effects of temperature on PM from barley roots using intrinsic protein fluorescence, spin probes, and fluorescence probes. They reported changes in slopes for ESR and fluorescence parameters at 12°C, but no significant change in the slope for ATP hydrolysis at 12°C was observed (Fig. 1B). Extremely accurate measurements might reveal changes in the slope at specific temperatures, but such a detailed analysis requires that the effect of temperature on K_m be considered (5, 7, 30), that linearity of the ATPase throughout the assay be verified at all temperatures, and that sufficient protein be used at low temperatures to accurately measure the ATPase rates. The object of the experiments in this paper was to determine the limitations of the assay procedures before making any detailed analyses. In general, the results for the PM ATPase resemble those from a previous analysis (7), although that study did not use a well-defined membrane preparation and the membrane fraction contained a Ca²⁺-phosphatase of high specific activity (discussed in DuPont and Hurkman, [10]).

The principal difference between the TN and PM ATPases of barley roots was that the Ea for the PM ATPase was much higher than the Ea for the TN ATPase. The Ea for ATP hydrolysis by the PM ATPase was 18 kcal/mol. This is similar to the results of Leonard and Hotchkiss (19), who obtained an estimate of 19 kcal/mol for the PM ATPase from corn

![Figure 11. Effect of temperature on movements of protons across tonoplast vesicles. A, Diagram of a right-side-out tonoplast vesicle, in which k_0 is the rate of H⁺ influx via the pump, k_1 is the sum of the rates of H⁺ efflux via antiports and symports, and k_p is the rate of H⁺ efflux by diffusion through the membrane; B, comparison of the effect of temperature upon two transport systems with different energies of activation and the same rate at 15°C. It is assumed that an Arrhenius plot of the activities is linear over the range of temperatures shown. The rate for both systems at 15°C is arbitrarily set at 0.5 unit/min and k_p has an Ea of 15 kcal/mol while k_0 has an Ea of 22 kcal/mol. The inset shows the same calculations plotted over a wider range of temperatures.](https://example.com/f11.png)
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roots for a temperature range of 15 to 38°C and O’Neill and Spanswick (22) who obtained an estimate of 17 kcal/mol for the PM ATPase of red beet for the temperature range of 20 to 30°C, but is somewhat higher than the results of Tu and Sliwiski (30), who estimated 14 kcal/mol for the PM ATPase from corn roots. The Ea for the TN ATPase from barley was approximately 10 kcal/mol, as was the Ea for the TN ATPase of tomato cells (11). The difference in Ea for the PM and TN ATPases may reflect differences in the structure and mechanism of action of the two enzymes. Differences in lipid composition may also play a role in the temperature dependence of the two ATPases. For example, the effects of temperature on the reconstituted PM ATPase of red beet were quite different from the effects of temperature on the native enzyme (22). If the TN ATPase has a lower Ea than the PM ATPase

The effect of temperature on the steady state ΔpH was quite different from the effect of temperature on initial rates of transport. For both the PM and TN ATPases there was a range of at least 10°C where the steady state ΔpH was relatively insensitive to temperature. One explanation might be that temperature had a similar effect on proton influx via the pump (Fig. 11, k₆) and on proton efflux (Fig. 11, k₊ + k₆) via antiports, symports, or passive diffusion through the membrane. Another possibility is that regulatory mechanisms adjust proton efflux so that a constant steady state ΔpH is achieved, regardless of the rate of proton transport by the ATPase. For instance, 500 μM vanadate had little effect on the steady state ΔpH formed by the PM ATPase, although it inhibited the initial rate of transport by 80% (12). Brauer et al. (4) made a detailed analysis of the effects of vanadate on the steady state ΔpH and demonstrated that, although vanadate inhibited influx of protons via the ATPase, it also greatly decreased proton efflux from the PM vesicles.

Rates of ATP hydrolysis declined at temperatures above 40°C for the TN ATPase and 45°C for the PM ATPase. Rates of proton transport and steady state ΔpH declined at lower temperatures, suggesting that the vesicles became ‘leaky’ before the ATPases were damaged by high temperatures. It is not simple to explain what leaky means in physical terms. One possibility is an increase in the number of vesicles that are not sealed and cannot form a ΔpH. If the vesicles remain sealed, then a decrease in steady state ΔpH with an increase in temperature means that the rate of the leak increased more than the rate of the pump, which may mean that there was a higher Ea for proton efflux than for proton influx (Fig. 11). There are several mechanisms to account for proton efflux from sealed vesicles. Simple diffusion through the lipid bilayer should have a low Ea, but diffusion of unaccompanied protons would be limited by the formation of a membrane potential. H⁺-efflux via proton antiports or symports might have a higher energy of activation than the ATPase. H⁺-efflux might also occur as a ‘back-leak’ via the pump. Not much data is available on the effects of temperature on active and passive processes in plant membranes (13, 16). Kami-ike et al. (16) examined the temperature dependence of the electrogenic pump of Chara corallina plasma membrane and found that the pump conductance was much more temperature dependent than the conductance of the passive channels. The Ea for permeation of urea and methylene into living cells was surprisingly high, ranging from 10 to 18 kcal/mol in Solanum slices (13).

More data on the effect of temperature on ion fluxes measured in isolated vesicles may help to disentangle the pathways and mechanisms involved in ion transport, particularly if methods to measure transport can be developed that are temperature insensitive. Excessively low or high environmental temperatures often limit plant growth and agricultural productivity. Membranes have been proposed to be sites of damage by chilling injury, freezing injury, and heat. Knowledge of the direct effects of temperature on isolated membrane vesicles will help to understand the effects of temperature on membranes in vivo.

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LITERATURE CITED


