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PROPERTIES OF A PLASMALEMMA ATPase OF THE MAIZE SCUTELLUM*

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Key Word Index—*Zea mays*; Gramineae; maize scutellum; phosphatase; ATPase; plasmalemma.

Abstract—Properties of a plasmalemma phosphatase of the maize scutellum, tentatively identified as an ATPase in a previous paper, were investigated. Fresh and frozen-thawed scutellum slices, that had been treated with 10 mM HCl to destroy acid phosphatases, were used as a source of enzyme. With the exceptions of the Na^+, K^+ and dinitrophenol experiments, the two kinds of slices gave similar results. ATP and CTP were the best substrates for the enzyme followed by TTP, UTP, CDP, ADP and GTP. UDP, nucleoside monophosphates, sugar phosphates, inorganic pyrophosphate and *p*-nitrophenyl phosphate were relatively ineffective as substrates. The K_m's for ATP and ADP were 0.65 and 5 mM, respectively, but the two substrates gave the same V_{max} (49.8 μmol P_i/hr/g slices). Previously, it was shown that the products of ATP hydrolysis are ADP, AMP and P_i. Using these previous results and from the time courses of ATP disappearance from the bathing solution and the appearance of P_i and ADP, it was concluded that ATP and ADP were hydrolysed by the same enzyme. The ATPase was not inhibited by oligomycin. *N-N'*-Dicyclohexylcarbodiimide (DCCD) was a poor inhibitor, and a water soluble analog of DCCD, 1-ethyl-3 (3 dimethylaminopropyl)-carbodiimide, gave only 33% inhibition. The relative effectiveness of divalent cations for stimulating ATPase activity was $Mn^{2+} > Mg^{2+} \geqslant Ca^{2+} > Co^{2+}$. Na^+ and K^+ gave a small additional stimulation in the presence of Mg^{2+}. However, Na^+ and K^+ gave a much greater stimulation when no divalent cation was added, and this occurred only when fresh slices were used. Dinitrophenol also increased ATPase activity only when fresh slices were used. Since it is likely that both the uptake of Na^+ and K^+ and the action of dinitrophenol would lower the electrochemical gradient of protons across the plasmalemma, the different results obtained with fresh slices indicate that the ATPase in these slices was under the constraint of a proton gradient.

INTRODUCTION

There are well-characterized ATPases (EC 3.6.1.5) in bacteria [1], mitochondria [2], chloroplasts [2] and animal cells [3–5]. Higher plant plasmalemma ATPases have also been reported, but they have not been as well characterized [6–9]. Studies with higher plant plasmalemma ATPases have been done primarily with partially purified membrane fractions and do not necessarily give much information about how the enzymes behave *in situ* [6–9]. ATPases are thought to be vectorial enzymes and their biological role cannot be clearly understood solely from studies with cell fractions or isolated membrane vesicles. For example, attempts to isolate intact right-side-out vesicles by osmotic lysis of spheroplasts have resulted in vesicles that are not comparable to the intact-cell plasmalemma in the orientation of certain plasmalemma-associated enzymes [10, 11].

Evidence for a tightly bound ATPase located on the plasmalemma of the maize scutellum was given in a previous paper [12]. The ATPase was considered to be a surface enzyme because when ATP was added to the solution bathing intact scutellum cells ADP, AMP and P_i appeared in the bathing solution and because the rate of reaction was sensitive to bathing solution pH and to added Mg^{2+}. This was confirmed by cytological studies with lead nitrate that showed ATPase activity located on the plasmalemma. ATPase was assayed using HCl-treated, fresh or frozen-thawed scutellum slices. The same level of ATPase activity and the same pH optimum were obtained with fresh and frozen-thawed slices. HCl treatment destroyed acid phosphatase and made possible a clear demonstration of a Mg^{2+} requirement [12].

This paper reports some of the properties of the scutellum plasmalemma ATPase in HCl-treated, fresh and frozen-thawed slices

RESULTS

Substrate specificity

Nucleotides, sugar phosphates, inorganic pyrophosphate (P-P_i) and *p*-nitrophenyl phosphate were tested as substrates for the plasmalemma ATPase (Table 1). CTP was hydrolysed at rates equal to that of ATP. The other nucleoside triphosphates were hydrolysed at rates 53–86% of that of ATP. ADP and CDP were good substrates, but UDP gave only 5–7% of the ATP activity. Nucleoside monophosphates, sugar phosphates, *p*-nitrophenyl phosphate and P-P_i were relatively ineffective as substrates. The greatest difference in relative phosphatase activity between the two kinds of slices was obtained when P-P_i was the substrate. P-P_i gave 34% of the ATP activity when frozen-thawed slices were used but only 10% when fresh slices were used.

Stoichiometry of the ATPase reaction

In a previous paper, it was shown that the products of ATP hydrolysis catalysed by HCl-treated scutellum slices were ADP, AMP and P_i [12]. Table 2 shows the initial

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Table 1. Substrate specificity of the plasmalemma ATPase*

Substrate	Activity (% of ATP) Frozen-thawed	Fresh
CTP	100	103
TTP	82	86
UTP	73	83
GTP	53	60
CDP	73	86
ADP	67	77
UDP	7	5
AMP	13	23
GMP	11	25
CMP	9	13
TMP	9	15
Glucose-6-P	14	27
Mannose-6-P	13	24
Inorganic pyrophosphate	34	10
p-Nitrophenyl phosphate	17	31

* Reactions were run with 3 mM substrate and 20 mM $MgCl_2$ for 1 hr. Each value is an average from 4 to 5 experiments.

Table 2. Stoichiometry of the ATPase reaction*

	ATP, μmol Fresh	ATP, μmol Frozen-thawed	ADP, μmol Fresh	ADP, μmol Frozen-thawed	P_i, μmol Fresh	P_i, μmol Frozen-thawed
Initial	51	50	0	0	0	0
60 min	24	26	11	11	46	38

* Initial reaction mixture contained 2.5 mM ATP and 20 mM $MgCl_2$. Each value is an average from 3 experiments.

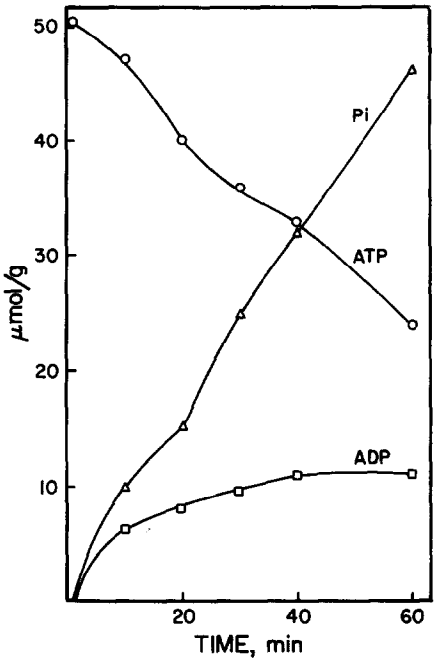

Fig. 1. Time courses of ATP disappearance from and ADP and P_i appearance in the bathing solution. Reactions were run with 2.5 mM ATP in buffered 20 mM $MgCl_2$. Each point is an average from 3 experiments. Standard deviations were less than 12% of the mean except for the first point on the P_i curve (16%) and on the ADP curve (22%).

and final (after 60 min) contents of ATP, ADP and P_i in the bathing solutions. AMP was not measured in these experiments. If ADP, AMP and P_i were the only products of the ATPase reaction, the amounts of P_i produced (Table 2) were 103–107% of the calculated amounts based on ATP disappearance. The time courses of ATP disappearance and ADP and P_i appearance in the solution bathing fresh slices are shown in Fig. 1; similar results were obtained with frozen-thawed slices (e.g. Table 2). As the amount of ATP in the bathing solution decreased, the amounts of ADP and P_i increased (Fig. 1). ADP increased rapidly during the first 10 min, but increased only slowly thereafter until it stopped increasing after 40 min. The P_i level, however, continued to increase. After about 20 min, the rate of P_i production rose and then remained linear. The ADP concentration did not rise above 0.55 mM during the course of the experiment while the ATP concentration fell from 2.5 mM to *ca* 1.2 mM.

Kinetics

The substrate concentration curve for ATP and the Lineweaver–Burk plots for ATP and ADP are shown in Fig. 2. The results obtained with fresh slices were almost identical to those obtained with frozen-thawed slices. The K_m's for ATP and ADP were 0.65 and 5.0 mM, respectively. ATP and ADP gave the same V_{max} (49.8 μmol P_i/hr/g). In these experiments, P_i formation from ATP was measured after only 10 min since it was apparent from the experiment of Fig. 1 that ADP also was being hydrolysed. Rates of P_i formation from ATP calculated from the first 10 min of the reaction were 33–45% greater than those calculated from a 1 hr reaction time (e.g. Fig. 1). In the experiment of Table 1, a 1 hr reaction time was used, and, as a result, relative rates of P_i production from ADP (relative to ATP) were higher than would be expected from the K_m and V_{max} values in Fig. 2.

Effect of divalent cations

Magnesium ions combine with ATP to form MgATP, which is the true substrate for ATPase [13]. The effects of $MgCl_2$ concentration of ATPase activities of fresh and frozen-thawed slices are shown in Fig. 3. The rate of reaction increased with increasing Mg^{2+} concentration at least up to 20 mM. This is far in excess of the ATP concentration of 3 mM. Storer and Cornish-Bowden [13] have shown that the true MgATP concentration cannot be derived by adding equimolar concentrations of Mg^{2+} and ATP. They suggested that in cases where the enzyme was not inhibited by high metal ion concentrations, 5 mM or more excess of Mg^{2+} over ATP was likely to give better stimulation. It was assumed in calculating the K_m's for ATP and ADP (Fig. 2) that in 20 mM $MgCl_2$ all of the nucleotide was present as the Mg^{2+} complex.

ATPase activity of the scutellum slices was also stimulated by Mn^{2+}, Ca^{2+} and Co^{2+} (Fig. 4). The relative effectiveness of the cations was $Mn^{2+} > Mg^{2+} \geqslant Ca^{2+} > Co^{2+}$. At high Mn^{2+} concentrations, the stimulatory effect was sharply decreased, ATPase activity at 20 mM Mn^{2+} being only one-half that obtained at 5 mM Mn^{2+} (Fig. 4). Calcium ions gave maximum stimulation at 10 mM, but at higher concentrations the stimulatory effect decreased. Cobalt ions were less effective than the other divalent cations tested, and maximum stimulation was obtained with 5–10 mM Co^{2+}.

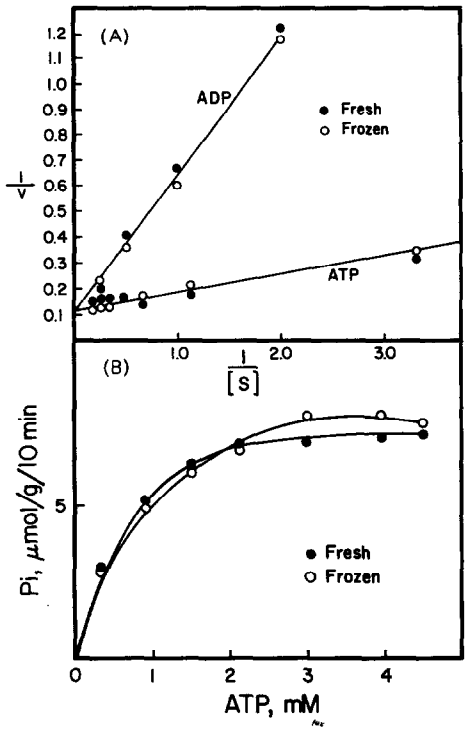

Fig. 2. Reaction rate as a function of substrate concentration. (A) Lineweaver–Burk plots for ATP and ADP. The ADP experiments were run for 30 min; but, for comparison with ATP, ADP rates were calculated for 10 min. Each point is an average from 4 experiments. v = μmol P_i/g/10 min; s = mM ATP or ADP. K_m's for ADP and ATP were calculated for each experiment, and the standard deviations were 2% or less of the mean K_m's. (B) Slices were replaced in buffered 20 mM $MgCl_2$ solutions containing different amounts of ATP, and the amounts of P_i liberated in 10 min were determined. Each point is an average from 2 experiments. Standard deviations were less than 5% of the mean.

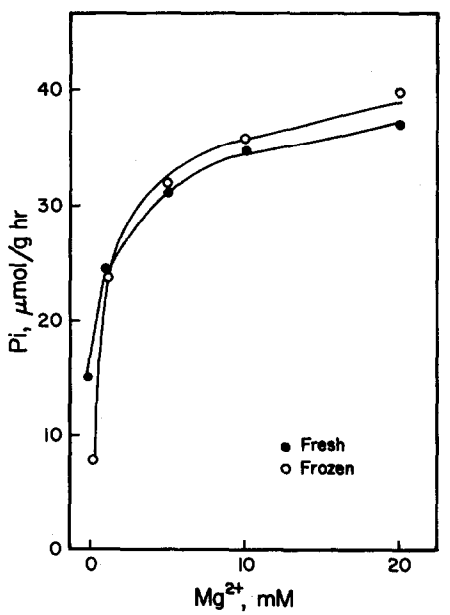

Fig. 3. Effect of $MgCl_2$ on ATPase activity. Reactions were run with 3 mM ATP for 60 min. Each point is an average from 3 experiments. Standard deviations were less than 11% of the mean.

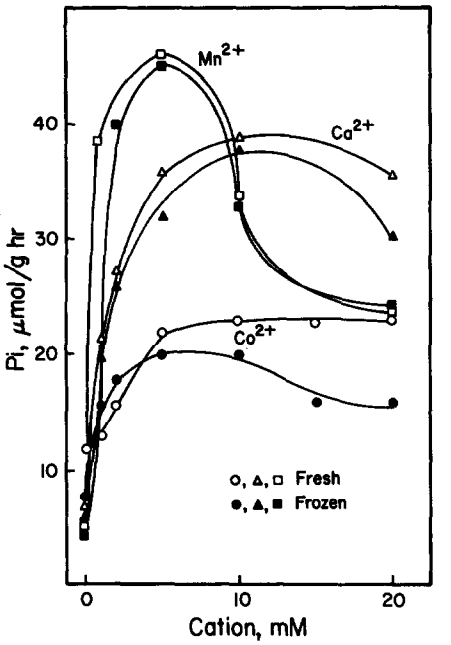

Fig. 4. Effect of $MnCl_2$, $CaCl_2$ and $CoCl_2$ on ATPase activity. Reactions were run with 3 mM ATP for 60 min. Each point is an average from 3 experiments. Standard deviations were less than 20% (for the Mn^{2+} curves, less than 10%) of the means.

Effect of Na^+ *and* K^+

Na^+ and K^+ were tested with and without added Mg^{2+} (Table 3). With fresh slices, the addition of Na^+ or K^+, alone or together in various concentration ratios, increased ATPase activity by 10–12 μmol P_i/g/hr (40–48%) when no Mg^{2+} was added and by only 1–5 μmol P_i/g/hr (2–11%) when Mg^{2+} was added. With frozen-thawed slices, Na^+ and K^+ increased ATPase activity by 3 μmol P_i/g/hr (50%) without added Mg^{2+} and 4–6 μmol P_i/g/hr (13–20%) when Mg^{2+} was added. ATPase activity was increased to a similar extent by either Na^+ or K^+ except when fresh slices were used with added Mg^{2+}, in which case K^+ but not Na^+ increased the activity (Table 3).

Table 3. Effect of Na^+ and K^+ on ATPase activity*

Na^+/K^+ ratio	Mg^{2+}	P_i, μmol/g/hr Fresh	P_i, μmol/g/hr Frozen-thawed
No Na^+ or K^+	+	45 ± 2.2	30 ± 2.0
	−	25 ± 5.6	6 ± 2.5
Na^+ only	+	46 ± 0	35 ± 0.8
	−	35 ± 0.6	9 ± 0.6
4	+	48 ± 0.8	34 ± 1.0
	−	35 ± 0.6	—
1.5	+	48 ± 0.8	36 ± 1.0
	−	36 ± 0.8	9 ± 0.6
0.25	+	50 ± 0.6	36 ± 0.6
	−	36 ± 1.0	9 ± 0
K^+ only	+	50 ± 0.6	36 ± 1.0
	−	37 ± 0.6	—

* Total monovalent cations equalled 50 mM, and the $MgCl_2$ concentration was 10 mM. Na^+- and K^+-free ATP (3 mM) was used in these experiments, and the MES buffer was brought to pH 6.5 with Tris base. Each value is an average from 3 to 4 experiments followed by the standard deviation.

Table 4. Test for inhibition of ATPase*

Inhibitor		% Inhibition Fresh	% Inhibition Frozen-thawed
Oligomycin	1 μg/ml	0	0
	10 μg/ml	0	0
DCCD	10 μM	17	20
	100 μM	9	10
	1 mM	4	7
EDCD	200 μM	11	4
	5 mM	29	22
	10 mM	33	30
NEM	10 μM	7	0
PCMB	500 μM	10	0
PCMBS	1 mM	14	9
	5 mM	32	—
Na azide	5 mM	4	2

* Reactions were run with 3 mM ATP and 20 mM $MgCl_2$ for 1 hr. Oligomycin and DCCD were made up in 95% EtOH, and controls received equal quantities of EtOH. The inhibitors were *N-N'*-dicyclohexylcarbodiimide (DCCD), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDCD), *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB) and *p*-chloromercuriphenyl sulfonic acid (PCMBS).

Inhibitors

Several enzyme inhibitors, some specific for ATPase, were tested with fresh and frozen-thawed slices (Table 4). In most cases, responses of fresh and frozen-thawed slices to the added inhibitors were quite similar. Although DCCD is a specific and potent inhibitor of some plant and animal ATPases, it did not greatly inhibit the ATPase of the scutellum slices. Maximum inhibition by DCCD was only 20%. A more water soluble analog of DCCD, EDCD, caused a 30–33% inhibition but only at a relatively high concentration (10 mM). A 5 mM concentration of PCMBS also gave a 32% inhibition. NEM, PCMB and sodium azide gave little or no inhibition, and oligomycin did not inhibit the ATPase.

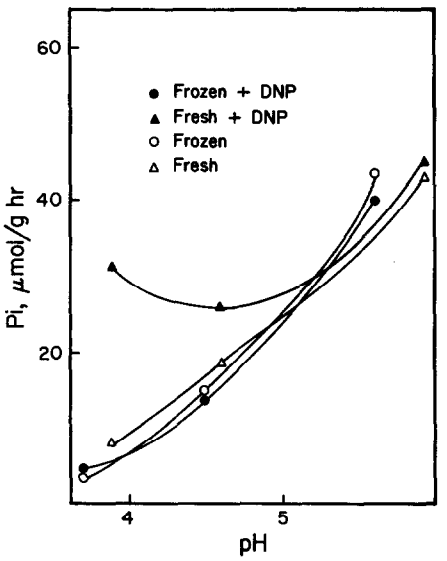

Fig. 5. Effect of DNP on ATPase activity. Reactions were run for 60 min with 3 mM ATP, 20 mM $MgCl_2$ and 50 mM MES, pH 3.5–6.0. DNP concentration was 0.5 mM. In the absence of ATP, DNP caused small amounts (*ca* 7 μmol/g) of P_i to leak from the slices, but the data reflect a corrected value. Each point is an average from 3 to 4 experiments. Standard deviations were less than 10% of the mean except for the first point on the 'frozen + DNP' curve which was 60%.

Effect of dinitrophenol (DNP)

In mitochondria [14], chloroplasts [15], bacteria [16] and intact ascites tumor cells [17], DNP stimulates ATPase activity. In fresh scutellum slices, DNP increased ATPase activity at pH's below 5 (Fig. 5). At pH 3.8, DNP caused a 4-fold increase in ATPase activity. In contrast to the results with fresh tissue, DNP had no effect on ATPase activity in frozen-thawed tissue. With H_2O- or HCl-treated fresh scutellum slices, DNP causes a proton influx and a sucrose efflux, but, as with ATPase stimulation, these occur only at pH's below 5 [18, 19]. Although the pH of the bathing solution affects the amount of DNP that enters the scutellum cells [18], it has a much greater effect on DNP-induced proton influx [18], on DNP-induced sucrose efflux [19] and on DNP stimulation of ATPase activity (Fig. 5).

DISCUSSION

Acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) have wide specificity and are not stimulated by metal ions [20, 21]. In contrast, ATPases show a more narrow specificity, primarily for nucleoside 5'-triphosphates [21], and are activated by certain cations, usually Mg^{2+} and Ca^{2+}. Some ATPases are additionally stimulated by Na^+ and K^+. The results of this paper and a previous paper [12] suggest that the phosphatase activity observed with HCl-treated, fresh and frozen-thawed scutellum slices is a plasmalemma-bound ATPase. The substrate specificities (Table 1), pH optimum [12], cation requirements (Figs. 3 and 4; Table 3), and K_m's (Fig. 2) are similar to those reported in the literature for plant plasmalemma ATPases [6, 7, 21–29].

The phosphatases of HCl-treated, fresh and frozen-thawed slices acted on a wide range of substrates (Table 1). ATP and CTP were the best substrates followed by TTP, UTP, CDP, ADP and GTP. Nucleoside monophosphates and sugar phosphates were relatively ineffective as substrates as was *p*-nitrophenyl phosphate, which is commonly used to detect acid and alkaline phosphatases [30]. These substrate preferences (Table 1) indicate the presence of an ATPase, but other phosphatases may also be present. The high activity obtained with ADP is similar to results obtained with isolated plant plasmalemma preparations [7, 25] and the presence of a specific nucleoside diphosphatase [31, 32] cannot be ruled out. However, the time courses of ATP disappearance and ADP and P_i appearance (Fig. 1) and the K_m's and V_{max} obtained with ATP and ADP (Fig. 2) indicate that ATP and ADP were hydrolysed by the same enzyme. The same V_{max} was obtained with ATP and ADP, but the K_m for ADP was *ca* 8-times greater than that for ATP. However, the data of Fig. 1 show that at high and nearly constant rates of ATP hydrolysis and P_i production, ADP production had nearly stopped. Thus AMP, the third product of ATP hydrolysis [12], was being produced at about the same rate that ATP was disappearing (i.e. 27 μmol/g/hr). During the course of the experiment (Fig. 1), the ADP concentration did not rise above 0.55 mM. This concentration would support a rate of ADP hydrolysis of only

ca 5 μmol/g/hr (from the experiment of Fig. 2) even in the absence of ATP. These results are interpreted to mean that following ATP hydrolysis, the ATPase did not release ADP but hydrolysed it to AMP. Since inorganic pyrophosphate was a poor substrate (Table 1), the ATPase must catalyse a sequential release of orthophosphate from the terminal pyrophosphate group of ATP.

Ca^{2+} was as effective as Mg^{2+} in activating the scutellum ATPase (Figs. 3 and 4). Mn^{2+} was the most effective cation, but, in contrast to Ca^{2+} and Mg^{2+}, Mn^{2+} produced a sharp maximum activation at a concentration of 5 mM (Fig. 4). The scutellum ATPase may be called a Mg^{2+}–Mg^{2+}–Ca^{2+} ATPase. Jolliot *et al.* [25] using potato tuber plasmalemma ATPase, obtained results with Mn^{2+} similar to those of Fig. 4 except that maximum activation was found at a concentration of 2 mM. With plasmalemma preparations from oat and maize roots, Mn^{2+} was nearly as effective as Mg^{2+}, but Ca^{2+} gave only 30% of the activity obtained with Mg^{2+} [7, 29].

Part of the cation stimulation of plasmalemma ATPase in fresh scutellum slices might be indirectly caused by cation uptake. An increased rate of proton efflux from roots during salt uptake is a well known phenomenon [e.g. 33], and proton efflux from fresh scutellum slices is greatly increased by exogenous Ca^{2+}, K^+ or Na^+ [34]. The stimulatory effect of cations on protein efflux is usually taken to mean that the proton pump (presumably, a reversible ATPase [35]) can produce a transmembrane electrical potential, inside negative [36]. The movement of cations into the cell would reduce the electrochemical gradient of protons against which the pump is working, thereby increasing the pumping rate. These ideas would explain why Na^+ and K^+ have a greater effect on ATPase in fresh than in frozen-thawed slices in the absence of exogenous Mg^{2+} (Table 3). However, in the presence of exogenous Mg^{2+}, the Na^+ and K^+ activation was small and was about the same in both fresh and in frozen-thawed slices. This activation may result from a direct effect of monovalent cations on the ATPase.

Inhibitor studies with ATPases from higher plants have given different results depending on the source of the enzyme, and the mechanisms of inhibition are poorly understood [7, 25, 29, 37–39]. The scutellum ATPase was not inhibited by oligomycin (Table 4). ATPases associated with plasmalemma and tonoplast preparations were reported to be insensitive to oligomycin although ATPases of mitochondria prepared from the same plant tissues were strongly inhibited [7, 25, 29, 38]. DCCD (10 μM) inhibited the scutellum ATPase by only 17–20%, and increasing the concentration 100-fold did not increase the degree of inhibition (Table 4). EDCD, a water soluble analog of DCCD, was also a poor inhibitor of the scutellum ATPase. DCCD (200 μM) almost completely inhibited plasmalemma ATPases from oat and maize roots [7, 29], whereas ATPases of the tonoplast of *Hippeastrum* petals [38] and the chloroplast envelope of spinach [39] were not inhibited by DCCD. However, the tonoplast ATPase was inhibited by EDCD [38]. Apparently, the scutellum ATPase is placed in the membrane in such a way that it is inaccessible to the inhibitors.

In the intact mitochondrion, the ATPase of the inner membrane is oriented in such a way that it reacts only with ATP of the matrix, and ATP hydrolysis is linked to proton transport out of the matrix. When the inner membrane is ruptured, the ATPase is no longer constrained by (coupled to) the electrochemical proton gradient and hydrolysis of exogenous ATP is rapid. The plasmalemma ATPase in intact cells of the maize scutellum, on the other hand, hydrolysed exogenous ATP, and disruption of the plasmalemma (by freezing and thawing [12]) caused little quantitative or qualitative change in ATPase activity. Although the rates of ATP hydrolysis were very similar in the two scutellum preparations, it is possible that significant amounts of ATPase were lost during freezing and thawing. The effect of DNP (Fig. 5) and the increased Na^+ and K^+ activation of the ATPase in intact cells (Table 3, and see above) indicate that the ATPase in fresh slices was under the constraint of a proton gradient. However, there is no evidence that hydrolysis of exogenous ATP caused proton transport in either direction across the plasmalemma. DNP, at pH's below 5, causes an efflux of sucrose out of and an influx of protons into the scutellum cell [18, 19] in addition to its effect on ATPase activity (Fig. 5). Presumably, both sucrose efflux and increased ATPase activity result from a lowering of the membrane electrical potential which accompanies proton influx.

EXPERIMENTAL

Preparation and treatment of scutellum slices. Maize grains (*Zea mays* L., cv McNair 508) were soaked in running H_2O for 24 hr, then placed on moist paper towels and grown in the dark at 24–25° for 72 hr. Scutella were excised and cut transversely with a razor blade into slices of 0.5 mm or less in thickness. Slices were washed in H_2O until the washings were clear, blotted on filter paper, and weighed into groups of 0.5 g. Slice samples (0.5 g) contained 80–90 slices, and each was 3–7 cells in thickness [12]. Both fresh and frozen slices were used. Slices were frozen at −4 to −5° and could be stored for at least 27 days with no loss of phosphatase activity. Fresh and frozen slices were incubated in 10 ml of 10 mM HCl for 1 hr at 30° before use in phosphatase assay. The bathing solns were replaced with fresh HCl after 30 min and at the end of the treatment slices were rinsed twice with H_2O. When frozen slices were used, HCl was added directly to the frozen slices.

Phosphatase assay. All incubations were carried out with 0.5 g slices in 10 ml of 50 mM MES buffer (pH 6.5) at 30°, except the DNP experiments in which the pH was varied. Substrates, cations and inhibitors were added at the concentrations shown in the tables and figs. The fresh slices did not take up P_i during the expts and the amounts of P_i leakage from either fresh or frozen-thawed slices were negligible [12]. Results are expressed as μmol of P_i, ADP or ATP formed or remaining in or disappearing from 20 ml of bathing soln containing 1 g of slices. P_i was measured by the modified Fiske–Subbarow method of ref. [40]. ATP was assayed by reacting it with glucose and hexokinase (EC 2.7.1.1) and measuring the G6P formed with G6P dehydrogenase (EC 1.1.1.49) [41]. ADP was assayed by reacting it with PEP and pyruvic kinase (EC 2.7.1.40) and measuring the pyruvate formed with lactic dehydrogenase (EC 1.1.1.27) [42].

Biochemicals. All biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. ATP, Sigma grade II, was used in this study [43]. Na^+- and K^+-free ATP was prepared by treating an ATP soln with Dowex-50, filtering and neutralizing the soln with Tris base.

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