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Studies on H^+-Translocating ATPases in Plants of Varying Resistance to Salinity[1]

II. K^+ STRONGLY PROMOTES DEVELOPMENT OF MEMBRANE POTENTIAL IN VESICLES FROM COTTON ROOTS

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ABSTRACT

Mg^{2+}-ATP-dependent H^+-translocation has been studied in membrane vesicles derived from the roots of *Gossypium hirsutum* L. var. Acala San Jose 2. Establishment of a positive membrane potential was followed by measuring SCN^- accumulation; establishment of ΔpH across the vesicle membranes by measuring quinacrine fluorescence quenching. High specificity for ATP was shown, and H^+-translocation was oligomycin stable. The pH profile for H^+-translocation showed an optimum at 5.5. The relationship between SCN^- accumulation and ATP concentration was approximately Michaelian; the apparent K_m was 0.7 millimolar. K-2-(*N*-morpholino)ethanesulfonic acid strongly promoted ATP-dependent SCN^- uptake (up to 180% stimulation). The effect was not given by Na-Mes. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone totally inhibited SCN^- accumulation, both in the presence and absence of K-2(*N*-morpholino)ethanesulfonic acid. Vanadate at 200 micromolar inhibited SCN^- uptake by about 10 to 40% in the absence of K^+, but more strongly in its presence (about 60%). NO_3^- at 100 millimolar inhibited initial rate of quinacrine quenching by about 25%. The NO_3^- insensitive fraction was activated by K^+; and inhibited by 200 micromolar vanadate to about 40%, provided K^+ was present. Saline conditions during the growth of the plants had no appreciable effect on the observed characteristics of H^+-translocation.

We have previously reported on proton-translocating ATPase activity in membrane vesicles isolated from the roots of the halophyte *Atriplex nummularia* (3). For purposes of comparison we have studied in parallel vesicles obtained from the glycophyte *Gossypium hirsutum* L. which, while far more sensitive to NaCl than the halophyte, is nevertheless ranked among crop plants which can withstand mild saline stress. Since we observed that saline conditions during the growth of the plant had marked effects on the properties of the proton translocating ATPase in the halophyte, we have examined the influence of growth conditions in the case of cotton as well. The NaCl concentration chosen in this case was 75 mM, a concentration which does not produce any deleterious effect on growth.

During the course of this study it has been observed that K-Mes promotes electrogenic H^+ transfer to an unusually high degree in membrane vesicles prepared from roots of this plant.

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MATERIALS AND METHODS

Gossypium hirsutum L. var. Acala San Jose 2 was grown hydroponically from seeds kindly supplied by Professor S. Izhar and Dr. A. Meiri of the Volcani Agricultural Research Centre.

Five d after germination in vermiculite the seedlings were transferred to aerated half-Hoagland and Snyder solution (5) with or without the addition of 75 mM NaCl, and placed in a light room (quantum flux density 300 $\mu E \cdot m^{-2}\ s^{-1}$, photoperiod 14 h light, 25°C). Roots were removed from 2-week-old plants, rinsed 3 times in ice-cold deionized H_2O or in 150 mM sucrose solution in the case of salt-grown roots to avoid osmotic shock (1). They were then ground in an ice-cold mortar with 4 volumes of homogenization medium containing 0.25 M sucrose, 2 mM EGTA, 5 mM $MgSO_4$, 1% BSA, 100 mM ascorbic acid, 3% (w/v) PVP, 25 mM BTP[2]-Mes, pH 7.2. The subsequent procedure for obtaining and fractionating membrane vesicles was as described for *Atriplex* (3), and the same pooled membrane fractions were used for the experiments. The transmembrane electric potential gradient (Δψ) and transmembrane ΔpH were measured with the aid of the labeled lipophyllic anion, $S^{14}CN^-$, and by following the quenching of quinacrine fluorescence, respectively.

For $S^{14}CN^-$ accumulation measurements aliquots of 50 μl vesicles, containing about 50 μg protein, were added in 10 μl assay medium containing 90 μM $KS^{14}CN$ (15,000 dpm; 2.5×10^{-4} MBq), 0.4 M sucrose, 30 mM $MgSO_4$, 0.5% BSA, and 10 mM BTP-Mes (pH 5.5). The vesicles were incubated with or without Tris-ATP as indicated in the figure legends. Unless otherwise indicated, the reaction was terminated after 10 min by dilution in 3.5 ml of a solution containing 0.4 M sucrose and 3 mM $MgSO_4$ (pH 7.0). The entire volume was then rapidly filtered, the vesicles retained by the filter were rinsed and counted by the same procedure as in Braun *et al.* (3).

A transmembrane ΔpH gradient was measured by following the quenching of quinacrine fluorescence, excitation wavelength 445 nm, emission wavelength 495 nm (conditions found to be optimal for measurement in our assay) in a Perkin-Elmer LS-5 luminescence spectrometer. Aliquots of 50 μl membrane fractions were incubated in 400 μl solution (final volume), containing 12.5 μM quinacrine, 100 mM BTP-Cl, 0.4 M sucrose, 5.0 mM $MgSO_4$, and 2 mM BTP-Mes (pH 7.0). The reaction was started by the addition of Tris-ATP to a final concentration of 5 mM.

Protein was determined in the membrane fractions by the method of Bradford (2).

[2] Abbreviations: BTP, bis-tris propane (1,3-bis[tris(hydroxymethyl)-methylaminol]-propane); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Cyt *c* oxidase activity was measured by the method of Hodges and Leonard (6).

KS[14]CN was obtained from the Radiochemical Center, Amersham, U.K. Quinacrine, sodium orthovanadate and polyvinyl polypyrrolidone were obtained from Sigma. Na_3ATP was obtained from Boehringer, Mannheim, West Germany, and was passed through a Tris-Dowex 50 column to obtain Tris-ATP.

RESULTS

Determination of $\Delta\psi$ and ΔpH in Membrane Vesicles. Evidence for the presence of an electrogenic proton-transferring ATPase in membrane vesicles prepared from cotton roots is supplied by the following observations: (a) provided that ATP is present, the vesicles accumulate SCN^- indicating the build-up of a transmembrane potential difference, inside positive (Fig. 1); (b) the build-up of a positive potential is also indicated by measurements of oxonol VI fluorescence quenching (not shown); (c) after the addition of BTP-Cl to collapse $\Delta\psi$, the establishment of a pH gradient across the vesicle membranes can be observed by following quenching of quinacrine fluorescence (Fig. 2); and (d) addition of the proton conductor FCCP[2] dissipates the ATP-dependent SCN^- accumulation (Table I).

The proton-pumping ATPase is stable to oligomycin (Table I) indicating that it is not the mitochondrial ATP synthase. This conclusion is supported by the fact that only a very low level of Cyt *c* oxidase activity was detected in the preparation. In a typical preparation Cyt *c* oxidase activity expressed per μg protein was only 3% of that observed in the mitochondrial fraction.

Table II shows that the ATPase displayed high specificity for ATP. Lack of utilization of IDP indicates that Golgi ATPase is not involved in the observed transport (10).

Dependence of Buildup of Membrane Potential on External pH. The pH profile for the establishment of the membrane potential was not appreciably affected by salinity conditions during the growing period, in strong contrast to our findings for the halophyte *Atriplex* (3). A peak was observed at about pH 5.5, whether the plants had been grown in the presence or absence of added NaCl (Fig. 3).

Accumulation of SCN^- as a Function of External ATP Concentration. We have earlier (3) presented reasons why the relationship between SCN^- accumulation and external ATP concentration might be expected to provide information with regard to the kinetics of the H^+-translocating ATPase. The data obtained

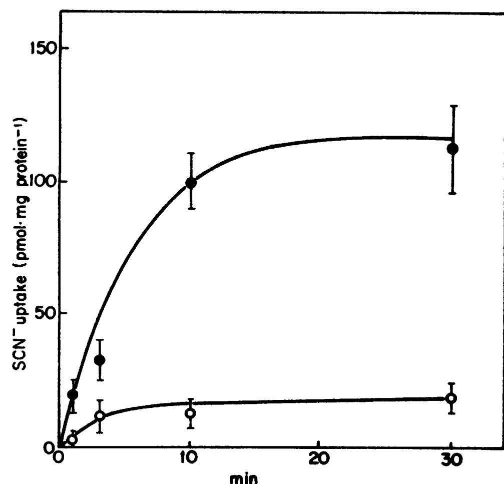

FIG. 1. Time course for accumulation of SCN^- in cotton root membrane vesicles in the presence (●) or absence (○) of 5 mM ATP. Medium as given in Methods, pH 5.5 Points give means of 4 replicates ± SE. Plants grown in absence of NaCl.

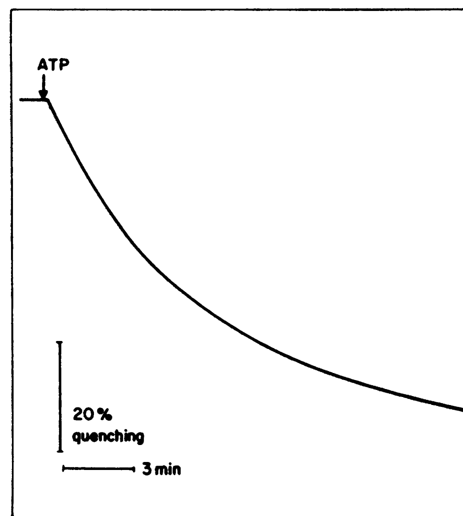

FIG. 2. Time course for quenching of quinacrine fluorescence after addition of ATP to medium containing cotton root membrane vesicles. Medium as given in "Materials and Methods" with the addition of 100 mM BTP-Cl, pH 7.0. Plants grown in absence of NaCl.

for SCN^- accumulation by cotton vesicles (Fig. 4) indicated an approximation to Michaelis-Menten kinetics (sometimes showing substrate inhibition at high ATP concentrations). By contrast the kinetics for uptake by *Atriplex* were non-Michaelian, particularly in the case of non-salt-grown plants (3). The apparent K_m calculated from the double reciprocal plot (Fig. 5) for non-salt-grown cotton plants was approximately 0.6 mM. Again in strong contrast to the results for *Atriplex*, growing the plants in saline medium had no marked effect either on the shape of the curve or on the apparent K_m, which was estimated from Figure 5 as approximately 0.7 for salt-grown plants.

Sensitivity to Vanadate and Nitrate. Vanadate sensitivity, regarded as a criterion for plasmalemma ATPase activity, was variable in our experiments. The percentage inhibition brought about by 200 μM vanadate ranged from 10 to 40% (Tables IV and V).

Partial inhibition by 100 mM NO_3^- was detectible by observing quinacrine fluorescence quenching after supply of BTP-Cl. Table V shows that initial slope was decreased by about 25%.

Effect of K^+ on the Establishment of $\Delta\psi$. Figures 6 and 7 show that addition of K-Mes to the incubation medium strongly promoted SCN^- accumulation. This was observed both for saline and non-saline-grown plants. The figures also show that the effect was limited to SCN^- uptake in the presence of ATP—supply of K^+ did not affect $\Delta\psi$ in its absence. Optimal K^+ concentration appeared to be 20 to 50 mM. The maximal stimulation (at optimal K^+ concentration) in the three experiments shown in Figure 7 was 180%, 53%, and 173%, respectively. Na-Mes did not produce an effect in this concentration range; neither did BTP-Mes (Table III).

A finding of interest was that vanadate sensitivity was higher in the presence of K^+ (Tables IV and V). For instance, in the experiments shown in Table IV, the inhibition of SCN^- accumulation by 200 μM vanadate ranged from 40 to 60% in the presence of 20 mM K-Mes, as compared with 10 to 36% in its absence. K-Mes stimulated quinacrine fluorescence quenching and increased its sensitivity to vanadate. Addition of 20 mM K-Mes stimulated the nitrate-stable quinacrine quenching by 26%; subsequent addition of vanadate inhibited the rate of quenching by 40% (Table V).

Addition of FCCP in the presence of K^+ completely dissipated the transmembrane electric potential (Table IV).

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Table I. *Effect of FCCP or Oligomycin on ATP-Dependent SCN^- Accumulation in Cotton Root Membrane Vesicles*

Medium as given in "Materials and Methods," pH 5.5. Uptake was measured after 10 min. Figures give the means of 4 replicates.

Treatment	ATP-Dependent SCN^- Accumulation	Percentage of Activity in Presence of ATP + Ethanol
	pmol·mg⁻¹ protein ± SE	
ATP (5 mM)	77.0 ± 9.6	
ATP (5 mM) + ethanol	53.9 ± 12.5	100
ATP (5 mM) + FCCP (10 μM)	3.8 ± 7.7	7
ATP (5 mM)	83.0 ± 15.6	
ATP (5 mM) + ethanol	71.0 ± 9.0	100
ATP (5 mM) + oligomycin (30 μg/mg protein)	63.1 ± 9.0	89

Table II. *Nucleotide Dependence of SCN^- Accumulation in Cotton Root Membrane Vesicles*

Medium as given in "Materials and Methods," pH 5.5. Uptake was measured after 10 min. Figures give means of 4 replicates.

Nucleotide Added	SCN^- Accumulation	Percentage of Activity in Presence of ATP
5 mM	*pmol·mg⁻¹ protein ± SE*	
0	21.2 ± 2.4	12
ATP	182.8 ± 7.9	100
ADP	25.5 ± 2.1	14
IDP	11.0 ± 1.9	6
GTP	19.1 ± 2.9	10

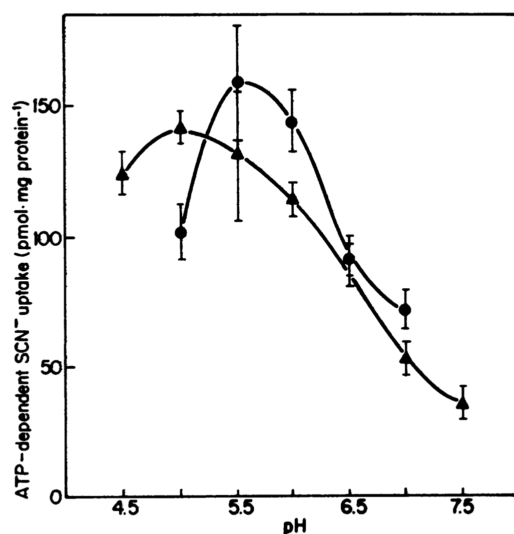

FIG. 3. pH profile for ATP-dependent accumulation of SCN^- in cotton root membrane vesicles. Media buffered with BTP-Mes-BTP-Hepes (20 mM). ATP concentration was 5 mM. Uptake measured after 10 min. Points give means of 4 replicates ± SE. Plants grown in presence (▲) or absence (●) of 75 mM NaCl.

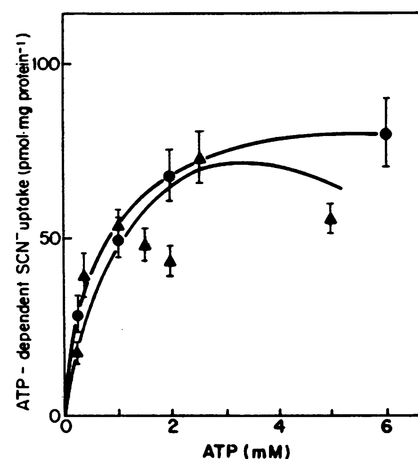

FIG. 4. ATP-dependent SCN^- accumulation by cotton root membrane vesicles (A), measured after 10 min, as a function of external ATP concentration. Medium as given in "Materials and Methods," pH 5.5. Points give means of 4 replicates ± SE. Plants grown in presence (▲) or absence (●) of 75 mM NaCl.

DISCUSSION

In contrast to our findings with the halophyte *Atriplex nummularia* (3) the degree of salinity during growth did not influence the characteristics of the proton-translocating ATPase activity observed in membrane vesicles from cotton roots. The pH profile, kinetics (SCN^- accumulation *versus* ATP concentration) and sensitivity to added K^+ were alike unaffected by growing the plants in 75 mM NaCl. (Compare the marked effect on these properties in *Atriplex* [3].) In the case of the halophyte, it appears that saline growth conditions were necessary to obtain levels of activity per mg protein of the order of that observed in cotton. Cotton vesicles showed relatively high activity in non-saline-grown plants and saline growth conditions produced no change.

Data for SCN^- accumulation may provide indications about the kinetics of the H^+-translocating ATPase (3). The near approach to Michaelis-Menten kinetics observed (Fig. 4) suggests that the carrier protein exists in fully active form and is not in need of ligand activation (7). Evidence discussed below leads to the conclusion that at least 2 translocating ATPases, tonoplast and plasmalemma, are present. The goodness of fit to Michaelian kinetics therefore suggests that the K_m for tonoplast transport must be close to that for plasmalemma transport.

The pH optimum observed (pH 5.5) is lower by 1 pH unit

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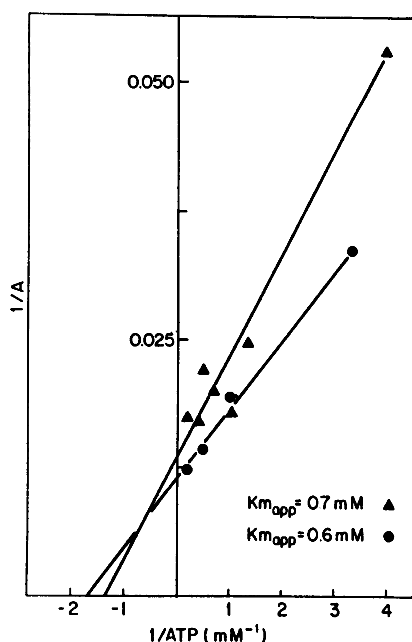

FIG. 5. Conditions as described in Figure 4. 1/A plotted against 1/[ATP]. Plants grown in presence (▲) or absence (●) of 75 mM NaCl.

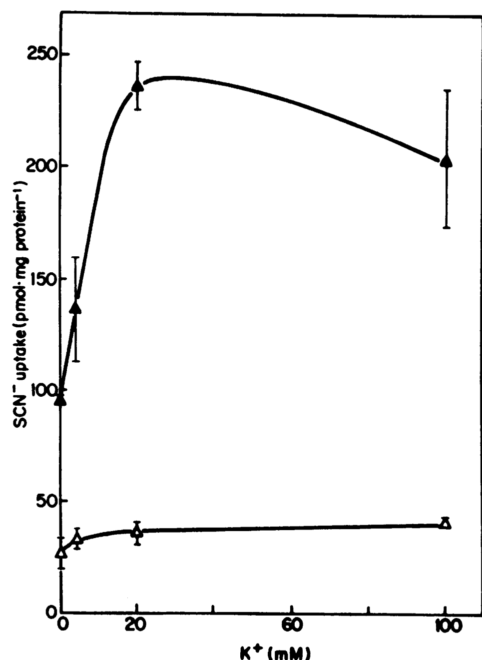

FIG. 6. Effect of increasing K^+ concentration on SCN^- accumulation in cotton root membrane vesicles in the presence (▲) or absence (△) of 5 mM ATP. K^+ supplied as K-Mes. Details of medium as in "Materials and Methods," pH 5.5. SCN^- uptake measured after 10 min. Points give means of 4 replicates ± SE. Plants grown in presence of 75 mM NaCl.

than that reported for the other plant species so far investigated (12).

An interesting feature of the cotton vesicles is the high degree of activation produced by adding K-Mes. Up to 180% stimulation of SCN^- accumulation was observed (Fig. 7). The possibility that K^+ penetration of the vesicle membranes was producing a K^+ diffusion potential can be discounted, since no effect of addition of K^+ was observed in the absence of ATP (Fig. 6). K^+

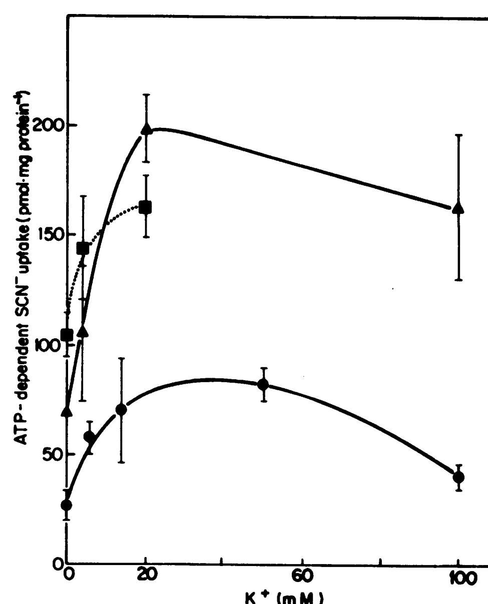

FIG. 7. ATP-dependent SCN^- accumulation in cotton root membrane vesicles as a function of external K^+ concentration. The three curves show three separate experiments. K^+ supplied as K-Mes. Details of medium as in "Materials and Methods," pH 5.5. ATP concentration was 5 mM. SCN^- uptake measured after 10 min. Points give means of four replicates ± SE. Plants grown in the presence (▲, ■) or absence (●) of 75 mM NaCl.

Table III. *Effect of Monovalent Cations on ATP-Dependent SCN^- Accumulation in Cotton Root Membrane Vesicles*

K^+, Na^+, and BTP^+ supplied as Mes salts, concentration 20 mM. Medium as given in "Materials and Methods," pH 5.5. ATP concentration was 5 mM. Uptake measured after 10 min. Figures give mean of 4 replicates.

Treatment	SCN^- Accumulation	
	Experiment I	Experiment II
	pmol·mg⁻¹ protein ± SE	
No ATP	10.5 ± 1.2	5.8 ± 0.9
ATP	115.3 ± 13.4	74.5 ± 5.2
ATP + K^+	176.4 ± 15.5	143.3 ± 4.5
ATP + Na^+	118.1 ± 12.2	76.6 ± 5.4
ATP + BTP^+	119.9 ± 11.8	77.7 ± 5.6

Table IV. *Effect of FCCP and Vanadate, in the Absence or Presence of K^+, on ATP-Dependent SCN^- Accumulation in Cotton Root Membrane Vesicles*

FCCP concentration was 10 μM; sodium orthovanadate concentration 200 μM. K^+ supplied as K-Mes, 20 mM. ATP concentration was 5 mM. Medium as given in "Materials and Methods," pH 5.5. Uptake was measured after 10 min. Figures give means of 4 replicates.

Treatment	SCN^- Accumulation	
	Experiment I	Experiment II
	pmol·mg⁻¹ protein ± SE	
No ATP	10.5 ± 1.2	5.8 ± 0.9
ATP	115.3 ± 13.4	74.5 ± 5.2
ATP + vanadate	105.0 ± 12.3	47.8 ± 3.3
ATP + K^+	176.4 ± 15.5	143.3 ± 4.5
ATP + K^+ + vanadate	108.4 ± 13.1	59.7 ± 5.7
ATP + ethanol	88.1 ± 10.7	59.8 ± 4.1
ATP + FCCP	15.3 ± 3.2	9.3 ± 2.4
ATP + K^+ + FCCP	16.5 ± 3.4	10.6 ± 2.5

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Table V. *Effect of Vanadate, of NO_3^-, and of Vanadate + NO_3^-, on Initial Rate of Quinacrine Quenching by Cotton Root Membrane Vesicles in the Presence or Absence of K*

Sodium orthovanadate concentration was 200 μM; BTP-NO_3^- concentration 100 mM; K^+ supplied as K-Mes at 20 mM. ATP concentration was 5 mM. Medium as given in "Materials and Methods," pH 7. The figures give means of 4 replicates.

Treatment	Initial Rate of Quinacrine Fluorescence Quenching
	arbitrary units·mg^{-1} protein min^{-1} ± SE
ATP + BTP-Cl	185 ± 21
ATP + BTP-Cl + K^+	180 ± 25
ATP + BTP-Cl + vanadate	174 ± 18
ATP + BTP-Cl + NO_3^-	138 ± 13
ATP + BTP-Cl + NO_3^- + K^+	174 ± 15
ATP + BTP-Cl + NO_3^- + K^+ + vanadate	99 ± 10

stimulation of H^+ transport has been reported in the literature only in one other case, that of radish seedling vesicles (11) but in the latter case stimulation of $\Delta\psi$ formation was very low (about 5%). Higher stimulation (about 80%) was observed on rate of ΔpH formation (11). A further interesting feature of the cotton vesicles was that in the presence of K^+, vanadate inhibition was markedly higher (Tables IV and V). The K^+ stimulation, and the sensitization by K^+ to vanadate inhibition, suggest that plasmalemma vesicles were present in the preparation. The NO_3^- sensitivity observed (Table V) indicates the presence of tonoplast vesicles as well (12). The fact that K^+ enhanced the sensitivity to vanadate may be partly attributable to an effect on dephosphorylation of the ATPase, as has been suggested for other systems (8). It may also have been partly due to the fact that in the presence of K^+ plasmalemma activity was contributing a larger fraction of the total activity observed.

Though K^+ stimulation of H^+-transport of the magnitude observed here has not been reported in the literature, stimulation of ATPase activity has frequently been noted and is regarded as a characteristic of plasmalemma ATPase activity (4). In most cases K^+ was applied together with a permeating anion, and the stimulation will therefore at least partially have been due to dissipation of the $\Delta\psi$ in sealed vesicles. The basis of the K^+ effect on Mg^{2+}-ATPase activity still awaits clarification.

It has been suggested (9) that stimulation by K^+ might be a nonspecific salt effect on enzyme conformation. In the present investigation, however, stimulation was observed for K-Mes, but promotion by Na-Mes in this concentration range was, if present at all, very slight. Various explanations might be suggested for a specific effect of K^+. K^+ might be a specific ligand activating the H^+-translocating ATPase; or it might itself be translocated contributing to the $\Delta\psi$ measured. No evidence for the latter possibility has been obtained in the present investigation, since FCCP completely dissipated the $\Delta\psi$ observed. Further work is required to clarify this large K^+ stimulation.

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