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ATP-DEPENDENT AND IONOPHORE-INDUCED PROTON TRANSLOCATION IN PEA STEM MICROSOMAL VESICLES

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The presence of an electrogenic pump in pea stem microsomal vesicles has already been demonstrated, but no evidence on the nature of the electrogenic ion has been presented (Rasi-Caldogno, F., De Michelis, M.I. and Pugliarello, M.C. (1981) Biochim. Biophys. Acta 642, 37–45). In this work we tested the usefulness of the ΔpH probe Acridine orange to monitor both ATP-dependent and ionophore-induced H^+ fluxes in pea stem microsomal vesicles. The H^+/K^+ exchanger nigericin causes a marked uptake of protons into the vesicles that can be followed, with similar results, both as Acridine orange absorbance changes and pH changes of the external medium. ATP induces an uptake of Acridine orange into the vesicles which is reversed by FCCP and abolished by the presence of Triton X-100 in the incubation medium, thus indicating an inward, ATP-driven, H^+ translocation. The ATP-dependent acridine orange uptake is Mg^{2+}-requiring and KCl-stimulated. Such activity is inhibited by two specific ATPase inhibitors, dicyclohexylcarbodiimide and diethylstilbestrol, while it is unaffected by oligomycin and Na_3VO_4. These results show that Acridine orange is a useful probe to measure pH gradients in our membrane system and are consistent with the hypothesis that an ATPase of plasmalemma may act as a proton pump.

Introduction

The mechanism of ion uptake in higher plants, although extensively studied, has not been clearly determined.

In recent years, a correlation between cation uptake and an Mg^{2+}-requiring, K^+-stimulated ATPase activity, associated with microsomal membrane fractions, has been established [1]. This observation led to the suggestion that an ATPase of plasma membranes may act as an ion pump, but the transport capabilities of such a pump have not been demonstrated. Indirect evidences, largely arising from 'in vivo' experiments, suggest that a pump, such as an ATPase capable of extruding protons, generates and maintains an electric potential (approx. 40–200 mV, interior negative) across the plasmalemma which, in turn, drives an ion uptake by plant cells [2–7].

To obtain a direct proof of this hypothesis it is necessary to demonstrate that isolated plasma membrane vesicles catalyze the ATP hydrolysis that lead to the generation of an electrical potential across the vesicle membranes by the proton translocating activity of an ATPase.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DES, diethylstilbestrol; BSA, bovine serum albumin; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt.

The presence of a proton pump in plasma membranes of *Neurospora crassa* [8–11] and yeast [12,13] has been clearly established. The study of transport phenomena in higher plant isolated plasma membrane vesicles has been attempted [14–17] and, recently, some direct evidence for the presence of a pump has been reported [18–24].

In a study of pea stem isolated plasma membrane vesicles, the demonstration that an ATPase is capable of generating a membrane potential has been reported [22]. It was shown that ATP hydrolysis, catalyzed by an ATPase located on the outer surface of functionally inverted plasma membrane vesicles, gives rise to an interior positive electric potential ($\Delta\psi$), measured as ATP-dependent [^{14}C]SCN^- uptake. Although it appears clear that pea stem plasma membranes catalyze an ATP-dependent charge translocation, no evidence on the nature of the electrogenic ion is given.

In this paper we report evidence for an ATP-dependent proton translocation in isolated pea stem plasma membrane vesicles monitored using the ΔpH probe Acridine orange. We also show that in such vesicles ionophore-induced H^+/K^+ fluxes occur. Such fluxes can be followed both by measuring acridine dye absorbance and pH changes of the incubation medium. A preliminary report of this paper has already been presented [20].

Materials and Methods

Plant material. Etiolated pea stem (*Pisum sativum* L., cv. Alaska) were grown in the dark on sand in a controlled environment (25°C temperature, 80% relative humidity) for 7 days.

50 g of etiolated pea stem were ground in 20 mM Hepes containing 5 mM $MgCl_2$; 0.4 M sucrose, 1 mM Na_2EDTA and 0.5% bovine serum albumin, pH 7.6, and then filtered through eight layers of gauze. The filtrate was centrifuged at $28000 \times g$ for 15 min. The supernatant was recentrifuged at $80000 \times g$ for 30 min. The pellet (microsomal membrane fraction) was resuspended in 10 mM Hepes, 5 mM $MgCl_2$, 0.4 M sucrose, 0.5% bovine serum albumin, pH 6.45 (approx. 25 g fresh weight/ml). This fraction possessed a high oligomycin-insensitive and Mg^{2+}-dependent, K^+-stimulated ATPase activity [25].

Acridine orange absorbance measurements. Acridine orange concentration was measured as absorbance change at 492 nm with 540 nm as the reference wavelength by an Aminco dual-wavelength spectrophotometer with magnetic stirring and thermostatic control (22°C).

Proton concentration measurements. Proton concentration was measured by an Orion Research pH meter connected to a recorder.

Protein assay. Protein concentration was measured by the biuret method described by Gornall et al. [26] using bovine serum albumin as a standard.

Results

Fig. 1 shows passive proton fluxes induced by ionophores on pea stem microsomal vesicles and followed by absorbance changes of the dye Acridine orange. A decrease of dye absorbance has been shown elsewhere to reflect an uptake of Acridine orange into vesicles, driven by a pH gradient across the membrane system: Acridine orange, following the classical pathway for weak bases, permeates the membrane vesicles in its unprotonated form, and is trapped inside in a protonated form [27–30]. The addition of the microsomal suspension to a medium without KCl caused a drop of absorbance of the dye (trace A) due to both the rapid uptake of Acridine orange in response to an inside acid pH gradient and, partially, to the turbidity of the added suspension. After an equilibration phase, the addition of nigericin induced a sharp decrease of the dye absorbance, as a consequence of H^+ translocation into the vesicles exchanged with endogenous K^+, that was reversed by ammonium sulfate. Trace B shows that the protonophore FCCP did not change the absorbance of Acridine orange, but, when the ionophore valinomycin was added to the medium, a drop of absorbance, similar to that caused by nigericin, was observed. When valinomycin was added before FCCP, a slight decrease of absorbance occurred (trace C), and the next addition of FCCP elicited a rapid decrease of absorbance, as in trace B experiment. The addition of nigericin to a medium containing 100 mM KCl caused an increase of absorbance, indicating a release of acridine dye from the interior of the vesicles to the medium and, therefore, a collapse of the H^+

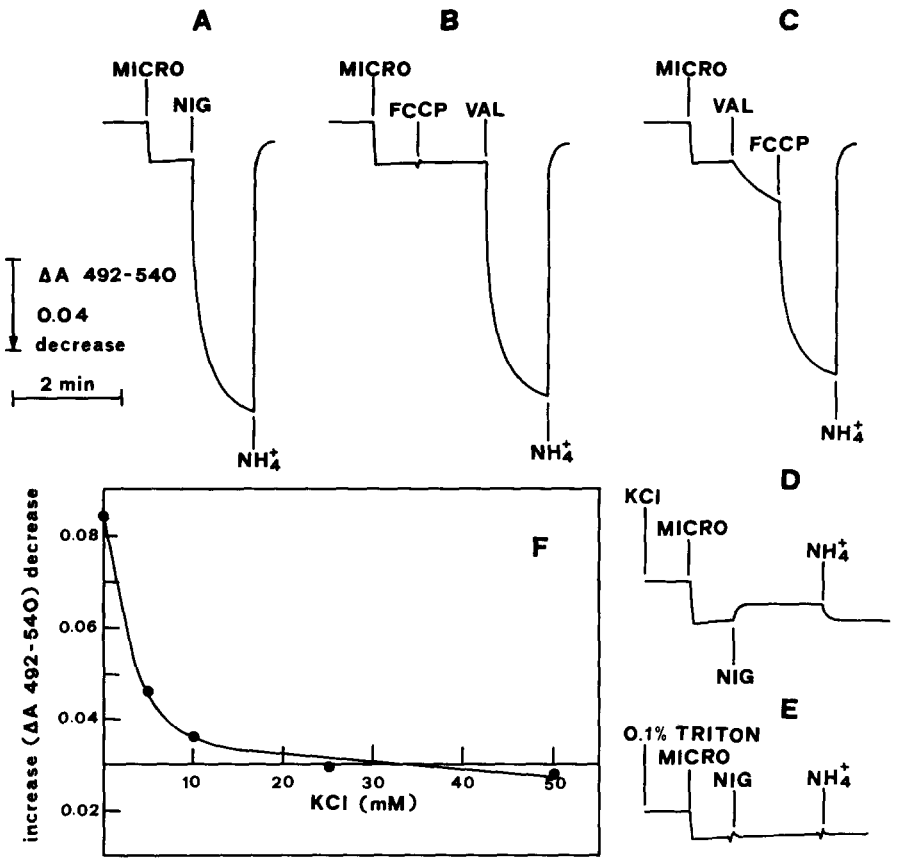


Fig. 1. Absorbance changes due to Acridine orange fluxes in pea stem microsomal vesicles. The medium contained: 10 μM Acridine orange, 10 mM Hepes, 5 mM $MgCl_2$, 0.4 M sucrose (pH 6.5) and 100 μl of microsomal suspension (1 mg protein) in a final volume of 2 ml. Traces A, B and C represent the decrease of absorbance induced by addition of 2 μg nigericin and 4 μg FCCP plus 4 μg valinomycin or vice versa, respectively. Traces D and E show nigericin-induced absorbance changes in the presence of 50 mM KCl or 0.1% Triton X-100. Insert F represents nigericin-induced Acridine orange movements at increasing KCl concentrations of the medium. In all traces nigericin-induced absorbance changes is collapsed by 10 μl of a saturated solution of $(NH_4)_2SO_4$.

gradient. The slight response of the dye to nigericin addition is related to the low pH gradient expected to be collapsed by nigericin; in fact the pH of the resuspending medium of the vesicles was approx. 6.45 and, hence, very near to that of the incubation medium (pH 6.5) (trace D). The relationship between acridine fluxes induced by nigericin and K^+ concentration in the medium is shown in insert F. The increase in KCl concentration induced a decrease of nigericin-mediated Acridine orange uptake. At approx. 25–30 mM KCl concentration no Acridine orange fluxes were observed, while at higher concentrations a release of the dye occurred. The presence of 0.1% Triton X-100 in the medium abolished the nigericin-induced dye movement (trace E).

The experiments with nigericin were also repeated after 30 min of pre-incubation of the vesicles in a K^+ free medium at 0°C. The effect of nigericin was similar to that found without pre-incubation, thus indicating that no leakage of K^+ from the vesicles took place during this period (data not shown).

To further check the reliability of the dye as a probe of pH gradients, the experiments of Fig. 1 were repeated using a glass electrode to measure the pH changes of a poorly buffered external medium. As can be seen in Fig. 2, the same pattern

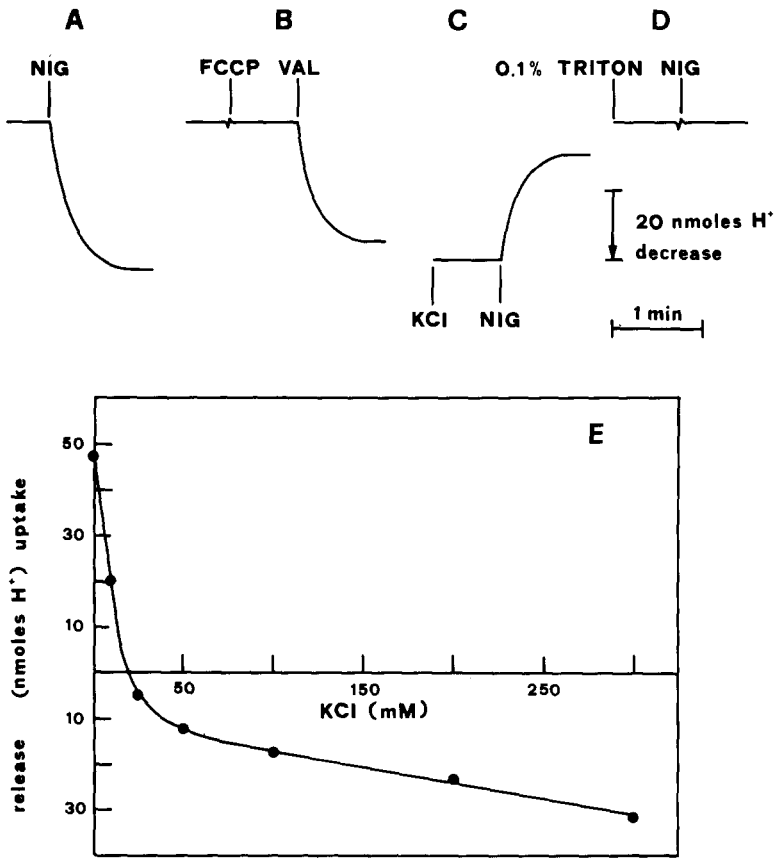
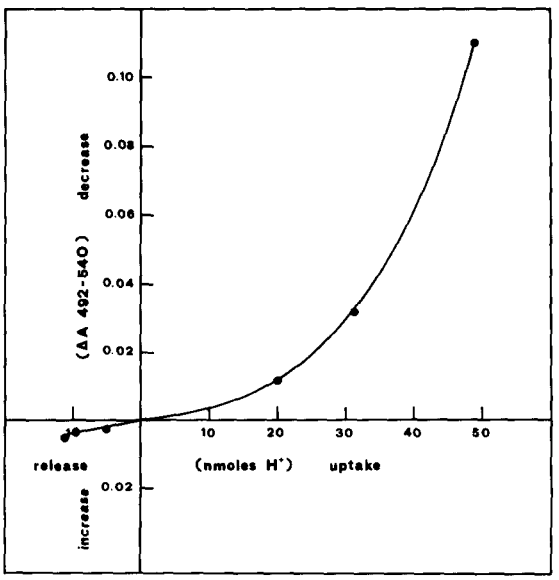


Fig. 2. pH changes due to proton fluxes in pea stem microsomal vesicles. The medium contained: 3 mM Hepes, 5 mM $MgCl_2$, 0.4 M sucrose (pH 6.5) and 100 μl of microsomal suspension (1 mg protein) in a final volume of 2 ml. pH changes after addition of 4 μg nigericin (trace A), 4 μg FCCP plus 4 μg valinomycin (trace B), 100 mM KCl (trace C) and 0.1% Triton X-100 (Trace D) are shown. Insert E represents nigericin-induced proton movements at increasing KCl concentrations of the medium.



of effects in response to the different additions was observed. In particular, the inversion of nigericin-induced H^+ fluxes at increasing KCl concentration occurred at approximately the same KCl concentration found in the Acridine orange experiments (insert E).

Using the data of Fig. 1 (insert F) and Fig. 2 (insert E) a calibration curve for the dye response versus proton translocation was built up (Fig. 3). The curve, crossing the origin of the axes, further supports the contention that a nigericin-mediated

Fig. 3. Correlation between Acridine orange movement and proton fluxes in pea stem microsomal vesicles. The curve was obtained by plotting the data of Fig. 1 (insert E) versus the data of Fig. 2 (insert F).

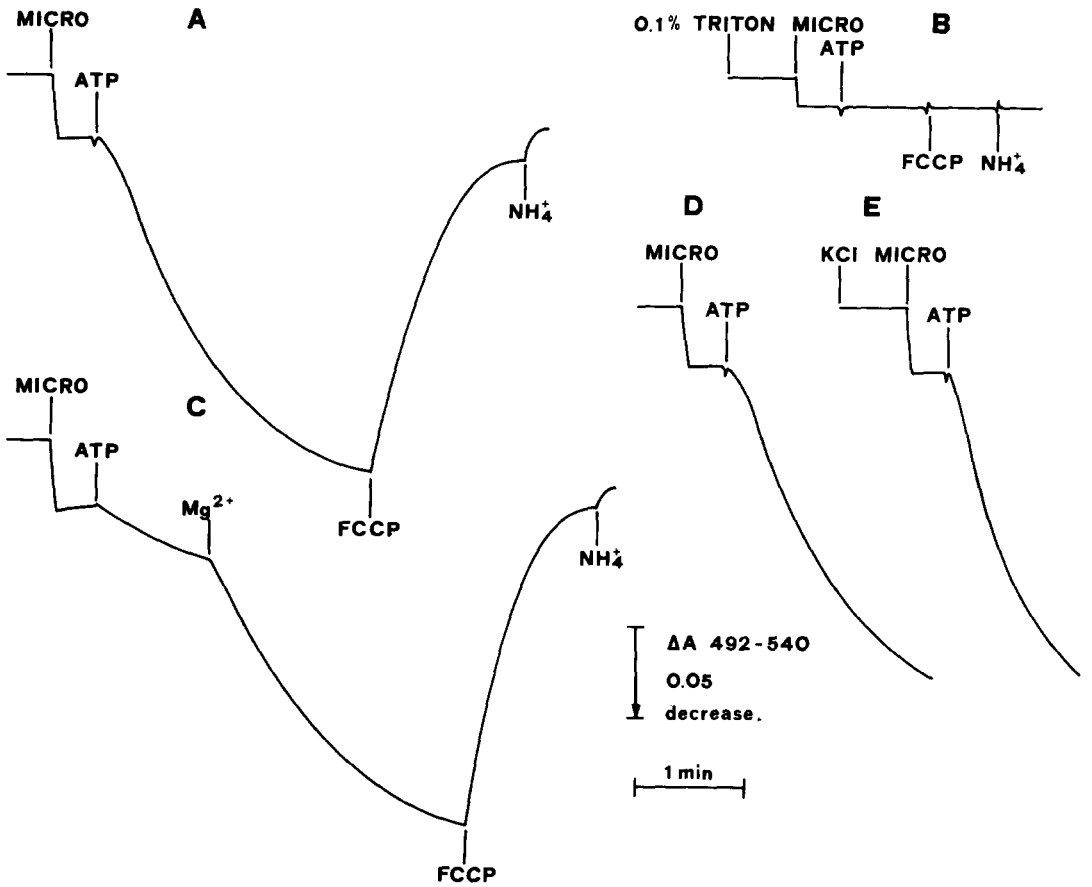


Fig. 4. ATP-dependent Acridine orange uptake in pea stem microsomal vesicles. The medium was as in Fig. 2, but, for the experiment relative to trace C, 5 mM $MgCl_2$ was replaced by 30 mM choline chloride. Trace A shows a decrease of absorbance caused by addition of 2.5 mM ATP that is reversed by 10 μg FCCP or abolished in the presence of 0.1% Triton X-100 in the incubation medium (trace B). Trace C shows ATP-dependent Acridine orange uptake in an Mg^{2+}-free medium and after addition of 5 mM $MgCl_2$ or $MgSO_4$. Traces D and E represent ATP-dependent Acridine orange uptake in the absence or presence of 30 mM KCl, respectively. Other additions: 150 μl of microsomal suspension (1.5 mg protein) and 10 μl of a saturated solution of $(NH_4)_2SO_4$.

proton uptake, expected to build up a pH gradient inside acid, can be monitored by a decrease of dye absorbance and, vice versa, a release is monitored by an increase of absorbance.

Evidence for the presence of an electrogenic pump in pea stem microsomal vesicles has already been reported [22]. However, no evidence was presented on the nature of the ion to be translocated. In order to achieve some insight on the identity of the electrogenic ion, we studied the effect of ATP on pH gradient in pea stem microsomal vesicles by the above method (Fig. 4). When ATP was added to the microsomal suspension, a marked decrease of absorbance of the dye was observed, indicating an ATP-dependent intravesicular acidification (trace A). The addition of FCCP dissipated such a gradient, and the next addition of ammonium sulfate collapsed the residual gradient. The ATP-dependent Acridine orange uptake was abolished by the presence in the incubation medium of 0.1% Triton X-100 (trace B). In an Mg^{2+} free medium the effect of ATP was markedly diminished, but the addition of 5 mM $MgCl_2$ or $MgSO_4$ restored the uptake of Acridine orange (trace C). Traces D and E show that the ATP-dependent dye uptake was stimulated 88% in the presence of 30 mM KCl.

Fig. 5 shows that the effect of ATP on Acridine

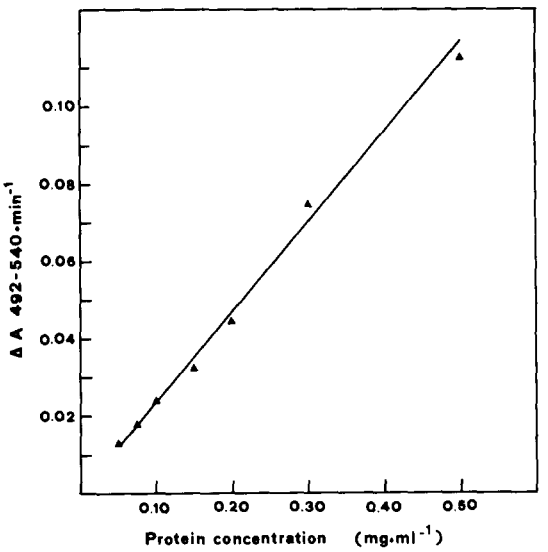


Fig. 5. ATP-dependent Acridine orange uptake at increasing protein concentration. Conditions as in Fig. 4.

orange uptake in microsomal vesicles increased with the increasing protein concentration.

The ATP-driven dye uptake was unaffected by 40 μM Na_3VO_4 and 4 μg/ml oligomycin (Fig. 6, trace B and C), while it was inhibited approx. 75% by 20 μg/ml DCCD or DES (traces D and E). Conversely, the latter two ATPase inhibitors had no effect on nigericin-mediated Acridine orange fluxes (data not shown).

Discussion

The usefulness of acridine dyes and, in particular, Acridine orange to measure pH gradients in animal vesicle systems has been discussed elsewhere [27–30]. Accordingly, we have found that Acridine orange is also an useful probe of pH gradients in pea stem microsomal vesicles. The observation that nigericin is able to induce ionic gradients in our

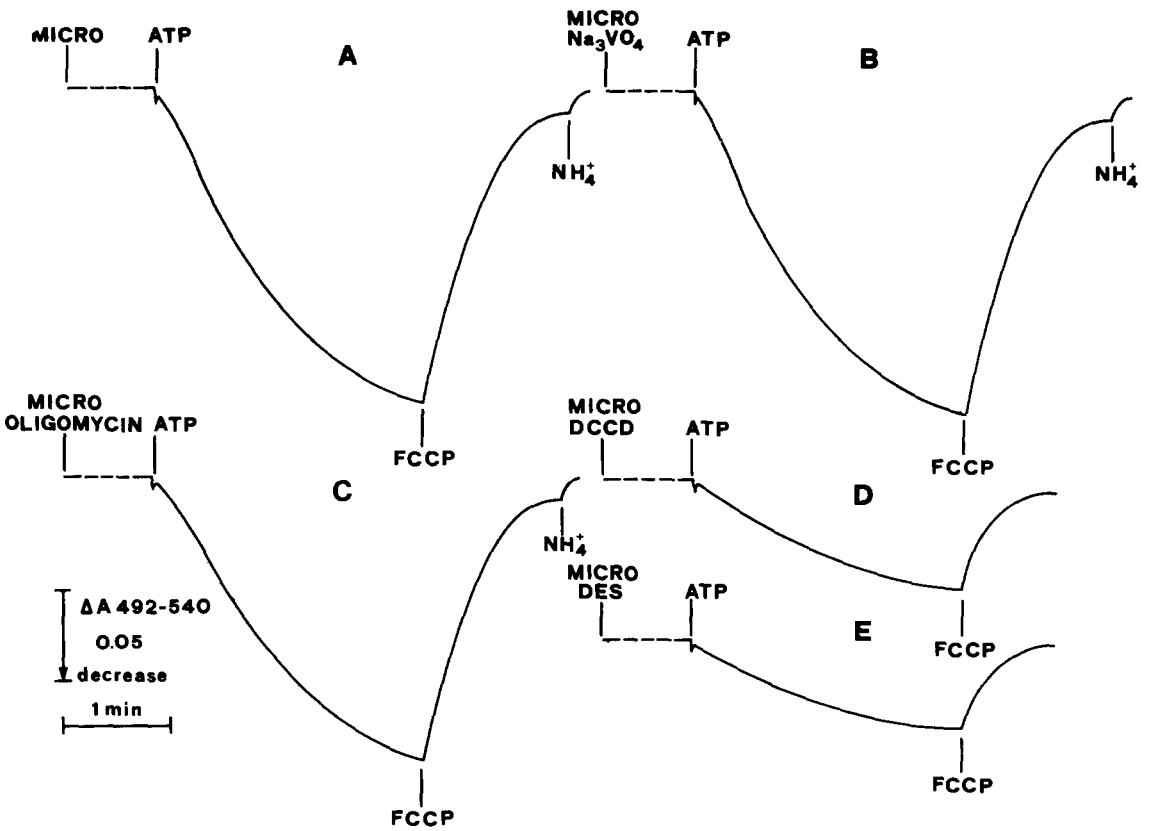


Fig. 6. Effect of ATPase inhibitors on ATP-dependent Acridine orange uptake. Conditions and additions as in Fig. 4. The vesicles were pre-incubated for 20 min in an ice bath in the presence of 40 μM Na_3VO_4, 4 μg/ml oligomycin, 20 μg/ml DCCD or DES, respectively, and after 5 min equilibration at 22°C, the reaction was started by adding 2.5 mM ATP.

pea stem microsomal preparation, as shown by two independent methods, indicates that our system contains predominantly sealed vesicles. The effect of nigericin is of the same magnitude, before and after 30 min of pre-incubation of the vesicles in the medium K^+ free used for dye uptake assay, thus indicating no leakage of K^+ during such a period. On the other hand, the retention of K^+ is not due to the absence of a permeant counterion, because the addition of FCCP, which would induce a proton permeability, does not cause any ion movement. Valinomycin alone causes a slight uptake of protons, probably due to a slight permeability to H^+ of our vesicle preparation, because the subsequent addition of FCCP restores the marked H^+/K^+ exchange induced by nigericin. On the basis of the above findings, we thus conclude that our pea stem microsomal vesicles show little, if any, permeability to H^+ and K^+ [31].

It has been previously shown that pea stem microsomes possess an electrogenic pump [22] that, in inverted vesicles, would take up electrophoretically a lipophylic anion, such as SCN^-, in response to a hypothesized primary electrogenic H^+ translocation. The results presented in this paper give direct evidence of an inward, ATP-driven, H^+ translocation. In fact, we have observed a marked uptake of the ΔpH probe Acridine orange into vesicles after the addition of ATP, indicating an interior acidification. An approximate evaluation of the efficiency of the pump can be attempted from the data in Fig. 3. The translocating activity results are of the order of 33 nmol H^+/mg protein per min translocated under the experimental conditions of Fig. 4. The ATP-dependent intravesicular acidification is Mg^{2+}-dependent and KCl stimulated, suggesting that such H^+ translocation is due to the activity of an Mg^{2+}-dependent, K^+-stimulated ATPase, resembling the proton pump which several authors consider central in ion transport of higher plants [5–7,32]. At this stage, however, we cannot exclude a role of the chloride anion in stimulating such an activity. The role of an ATPase pump is shown by the observation that the H^+ gradient is collapsed by FCCP and inhibited by the two specific plasmalemma ATPase inhibitors, DCCD and DES. Conversely, such a proton pump is insensitive to oligomycin and vanadate, thus appearing, for such features, similar to that found by Du Pont et al. [21] in maize roots, rather than that of *Neurospora* plasma membranes [9].

The present results, together with the recent findings of other authors [18–24], provide direct evidence that the proton pump in higher plants is an Mg^{2+}-dependent ATPase capable of translocating protons. Hence, the generation of a membrane potential [22] might be linked to proton translocation. In this regard, since FCCP reverses the ATP-dependent proton uptake and is ineffective on passive proton fluxes, we suggest that the pump may be electrogenic. The nature of the ion which is cotransported with H^+ is still to be clarified.

The H^+ translocating activity of our pea stem microsomal ATPase is linked to the presence of 0.5% bovine serum albumin in the extraction and resuspending media, which seems to increase the stability of our membrane vesicle system. In fact, the H^+ translocating activity recovered in the experiments presented in this paper is higher than that found using 0.1% bovine serum albumin [20].

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