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Ethanol-Induced Activation of ATP-Dependent Proton Extrusion in *Elodea densa* Leaves[1]

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ABSTRACT

In *Elodea densa* leaves, ethanol up to 0.17 M stimulates H^+ extrusion activity. This effect is strictly dependent on the presence of K^+ in the medium and is suppressed by the presence of the plasmalemma H^+-ATPase inhibitor vanadate. Stimulation of H^+ extrusion is associated with (a) a decrease in cellular ATP level, (b) a marked hyperpolarization of transmembrane electrical potential, and (c) an increase in net K^+ influx. These results suggest that ethanol-induced H^+ extrusion is mediated by an activation of the plasma membrane ATP-dependent, electrogenic proton pump. This stimulating effect is associated with an increase of cell sap pH and of the capacity to take up the weak acid 5,5-dimethyloxazolidine-2,4-dione, which is interpretable as due to an increase of cytosolic pH. This indicates that the stimulation of H^+ extrusion by ethanol does not depend on a cytosolic acidification by products of ethanol metabolism. The similarity of the effects of ethanol and those of photosynthesis on proton pump activity in *E. densa* leaves suggests that a common metabolic situation is responsible for the activation of the ATP-dependent H^+-extruding mechanism.

ATP-dependent H^+ extrusion is regulated in vivo by various factors, including cytosolic pH (8, 21), E_m[2] (16, 22), and natural endogenous and exogenous factors such as hormones and phytotoxins (13). More recently the conclusion that the stimulating effect of light on H^+ extrusion in *Elodea* leaves might be mediated by photosynthesis-induced metabolic changes (14) has emphasized the need to understand the relationship between metabolism and activity of the H^+ pump.

In search of factors that may influence the proton pump of *Elodea densa* leaves, we have focused our attention on certain glycolytic metabolites including sugars, organic acids, glycerol, and ethanol. Among these, ethanol was found to induce in *E. densa* leaves a marked, reproducible stimulation of the capacity to acidify the medium.

A stimulating effect of ethanol on the reduction of extracellular electron acceptors and on the H^+ extrusion associated with it has been reported by Craig and Crane (6), Chalmers et al. (4), and Böttger and Lüthen (3), and it has been interpreted as being due to the alcohol dehydrogenase-mediated rise of NADH, as an electron donor to the plasma membrane redox system (10). A stimulation of H^+ extrusion by ethanol and proponol in the absence of artificial extracellular electron acceptors has been reported by Böttger and Lüthen (3).

Moving from these results, the main objectives of the present investigations were (a) to obtain a general description of the effect of ethanol on H^+ extrusion in *E. densa* leaves, (b) to ascertain whether it is mediated by the ATP-driven proton pump, and (c) to acquire some preliminary information concerning its mechanism: in particular, whether or not the stimulation of H^+ extrusion by ethanol might be due to cytosol acidification and/or E_m depolarization (as seen in the case of ferricyanide reduction by the plasma membrane redox system) (12, 19, 28).

MATERIALS AND METHODS

Elodea densa plants were cultivated in large tanks in a greenhouse (19). Actively growing shoots (about 5 cm long) were collected in the morning and kept under running tap water for about 1 h. The leaves were then excised, randomized, and pretreated for 2 h in 0.5 mM $CaSO_4$ in the dark at 20°C in agitated Erlenmeyer flasks (18 leaves = 150 mg fresh weight in 10 mL) and then treated as described in detail for each experiment. When required, FC, Na-orthovanadate, and DCMU were added to the $CaSO_4$ solution 30 min before the experiment.

pH and H^+ Extrusion Measurements

After pretreatment, samples (150 mg fresh weight/7 mL) were transferred to a solution containing 0.5 mM Mes (pH 5.5 with BTP), 0.5 mM $CaSO_4$, 1 mM K_2SO_4. Variations in the tested solutions are indicated in figure legends. The experiments were conducted in triplicate at 20°C either in the dark (in the presence of 5 μM DCMU) or in the light (50 W/m^2). Proton extrusion was measured at different times by back-titration of the external medium after removal of CO_2 according to Lado et al. (11). The titration curves (between pH 4 and 7) were also used to measure eventual changes of the buffering capacity of the medium due to the release of weak acids from the leaves.

K^+ Uptake Measurements

Changes in K^+ concentration in the medium were determined by atomic absorption spectrophotometry (Varian

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[2] Abbreviations: E_m, transmembrane electrical potential; FC, fusicoccin; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)-propane; DMO, 5,5-dimethyloxazolidine-2,4-dione; K^+_o, extracellular K^+.

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Techtron AA 1275). Basal medium contained 10 mM Mes (pH 5.5 with BTP), 0.5 mM $CaSO_4$, 0.25 mM K_2SO_4, 5 μM DCMU with and without 0.2 mM Na-orthovanadate.

E_m Difference

Measurements of E_m were performed by the microelectrode procedure described by Marrè et al. (17). The basal medium contained 1 mM Mes (pH 5.5 with BTP), 0.5 mM $CaSO_4$, 1 mM K_2SO_4, 5 μM DCMU. Variations in the medium composition are described in the legends to the figures.

Cell Sap pH

When treatments were terminated, leaves were washed in distilled water, blotted on filter paper, transferred into plastic syringes, and frozen at −30°C for at least 3 h. After freezing and thawing, the cell sap (about 0.2 mL) was pressed out of the syringe and the pH was measured by means of a Radiometer pH meter equipped with a flat tip electrode (Ingold 40424, lot 40330-M8).

Accumulation of DMO and Evaluation of Cytoplasmic pH

The reliability of the weak acid-weak base distribution method for the evaluation of cytosolic and vacuolar pH in *Elodea* leaves has been demonstrated by Beffagna and Romani (2). In our experiments, the weak acid used as a probe was [2-^{14}C]DMO (155 kBq/μmol). For DMO accumulation after pretreatments, the samples (150 mg fresh weight) were transferred to 10 mL of a solution containing 10 mM Mes (pH 5.5 with BTP), 0.5 mM $CaSO_4$, 5 μM DCMU, 5 μM DMO, 1% (v/v) ethanol (in the presence or absence of 1 mM K_2SO_4). At the end of the incubation with DMO, the leaves were rinsed with the corresponding solution without label for 3 min at 0°C, then washed with water, blotted dry, and placed into the scintillation vials for tissue digestion and bleaching prior to determination of their radioactivity.

The pH values of "bulk cytoplasm" were calculated from the accumulation of DMO by the weak acid distribution method as described by Beffagna and Romani (2). The pH values had been calculated utilizing the previously measured cell sap pH value as vacuolar pH and assuming that DMO distribution had reached equilibrium after 90 min of treatment.

Determination of Metabolites

After pretreatment, samples (150 mg fresh weight) were transferred to 7 mL of a solution containing 10 mM Mes/BTP buffer (pH 5.5), 0.5 mM $CaSO_4$, 5 μM DCMU, 0.1 mM FC, 1% ethanol (with or without 1 mM K_2SO_4). At the end of treatments, leaves were washed in distilled water, blotted on filter paper, and frozen in liquid nitrogen. Malate was determined enzymically by the malate dehydrogenase/citrate synthase method as described by Stitt et al. (29). The intracellular content of ATP was measured by the luciferin/luciferase method as described by Novacky et al. (24).

All the experiments were run in triplicate and repeated at least three times. The data presented are those of a typical experiment in which the coefficient of variation did not exceed ± 6%.

RESULTS

Effects of Some Metabolites and Alcohols on External pH

In a preliminary series of experiments, we measured the effects of some metabolites and alcohols on H^+ extrusion, measured by titration after 60 min of incubation in a medium containing 1 mM K_2SO_4, 0.5 mM Mes-BTP, pH 5.5, 0.5 mM $CaSO_4$, 150 mg fresh weight of leaves in 7 mL. Among the compounds tested, 0.17 M ethanol and propanol induced a well-marked stimulation of H^+ extrusion, whereas at the same concentration, methanol, buthanol, and isopropanol had no significant effect, and pentanol induced an alkalinization of the medium. Fifty millimolar glucose and 0.2 M glycerol were ineffective (data not shown).

Characterization of the Effects of Ethanol on H^+ Extrusion

As shown in Figure 1, ethanol, in the presence of 1 mM K^+, markedly stimulated H^+ extrusion. The maximum effect was observed for the 0.17 M concentration, already significant after 15 min of treatment, and increased roughly linearly with time. The titration curves of the incubation media between pH 4 and 7 indicated that ethanol, up to 0.5 M, did not induce any significant difference in the buffer capacity of the medium (e.g. by acetic acid arising from ethanol oxidation via alcohol dehydrogenase). This ruled out the possibility of an alcohol-induced release of organic acids by the leaves. At ethanol concentrations higher than 0.17 M, the stimulating effect decreased to become strongly inhibitory at the 1.7 M concentration. The absence of K^+_o completely suppressed H^+ extrusion both in the controls and in the ethanol-treated samples (Fig. 2). Because ATP-dependent proton extrusion requires the presence of K^+ (or of other permeant, depolarizing cations) in the medium (15, 16, 22), this finding suggested an involvement of the ATP-dependent

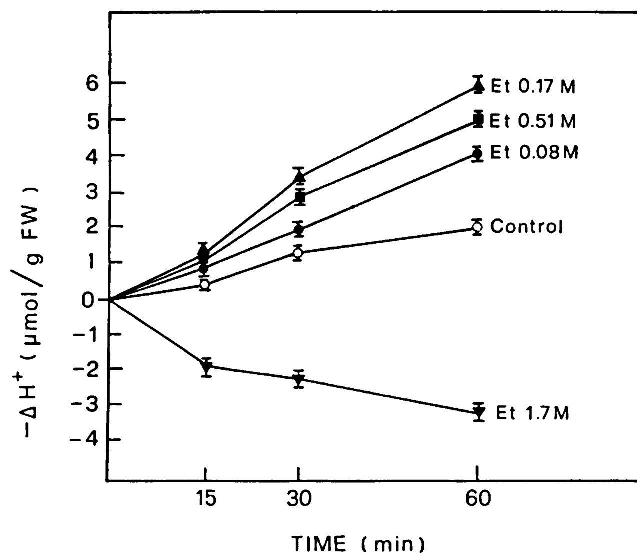

Figure 1. Effect of increasing concentrations of ethanol (Et) on apparent H^+ extrusion ($-\Delta H^+$). Control: 0.5 mM Mes/BTP, pH 5.50, 0.5 mM $CaSO_4$, 1 mM K_2SO_4, 5 μM DCMU. Bars indicate ± SE.

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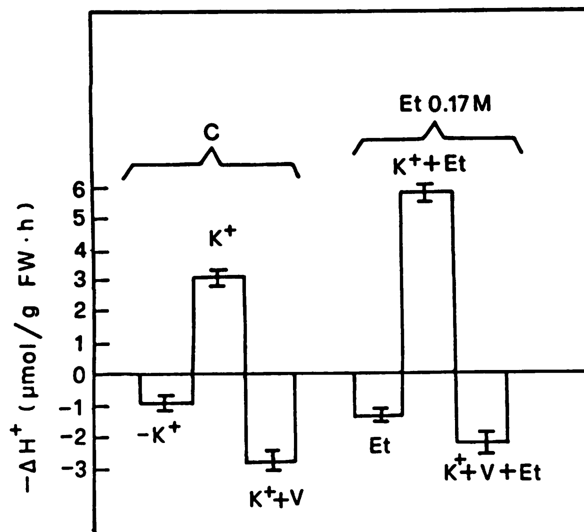

Figure 2. Effect of K^+ and vanadate on ethanol-induced H^+ extrusion. Control: 0.5 mM Mes/BTP, pH 5.5, 0.5 mM $CaSO_4$, 5 μM DCMU. When present: 1 mM K_2SO_4, 0.2 mM vanadate (V), 0.17 M ethanol (Et). Bars indicate ± SE.

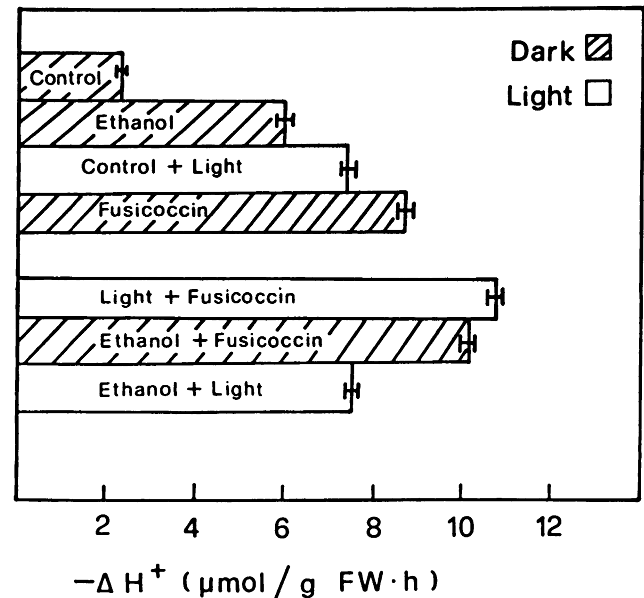

Figure 3. Effect of FC and light on basal- and ethanol-induced H^+ extrusion. Basal medium (control): 0.5 mM Mes/BTP pH 5.5, 0.5 mM $CaSO_4$. When present: 1 mM K_2SO_4, 0.17 M ethanol, 0.1 mM FC. dark, 5 μM DCMU in the medium; light, 50 W/m². Bars indicate ± SE.

H^+-ATPase. This hypothesis is supported by results reported in Figure 2, showing that 0.2 mM vanadate, a relatively specific inhibitor of the plasmalemma H^+-ATPase (see ref. 1), completely suppressed both the basal and the ethanol-stimulated H^+ extrusion.

Interactions between Ethanol and FC and between Ethanol and Light in their Effect on H^+ Extrusion in *Elodea* Leaves

The analysis of the interactions among different factors influencing a single process might be of help in understanding their mechanism of action. A further series of experiments was thus aimed at defining the interactions between ethanol and other factors known to influence H^+ extrusion in *Elodea* leaves, such as FC and light.

Previous results had shown that FC and light are somewhat, although not completely, additive in promoting H^+ extrusion (14). The data reported in Figure 3 confirm this result and also show that some additivity is observed between the effects of FC and ethanol, whereas no additivity at all is seen between those of ethanol and light. In fact, in our conditions the light-induced stimulation of K^+-dependent H^+ extrusion was not further enhanced by the presence of ethanol.

Changes of E_m and of K^+ Uptake Associated with Ethanol-Induced H^+ Extrusion

As shown in Figure 4, the addition of 0.17 M ethanol induced a significant hyperpolarization (35–45 mV) of E_m, already detectable after a few minutes of treatment and reaching its maximum after about 15 min. This ethanol-induced hyperpolarization was completely reversed by washing and was suppressed by the addition of the protonophore uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and by the presence of the H^+-ATPase inhibitor, vana-

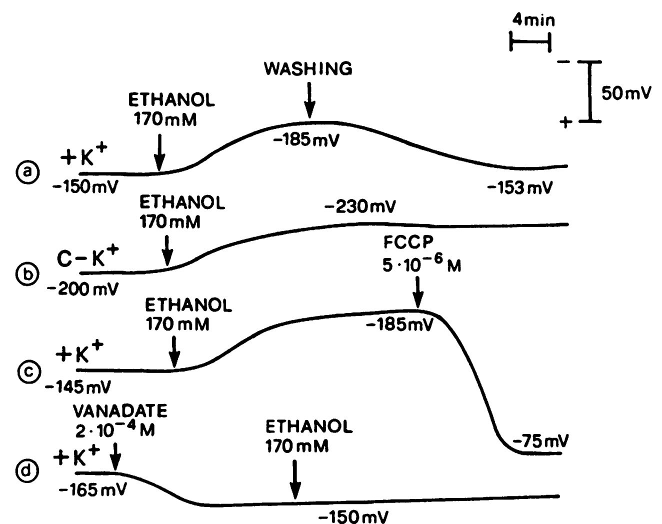

Figure 4. Ethanol-induced changes in membrane potential. Two leaves were maintained in an aerated solution, thermoregulated at 20°C (continuous flow 15 mL/min). C-K^+, 1 mM Mes/BTP, pH 5.5, 0.5 mM $CaSO_4$, 5 μM DCMU; +K^+, + 1 mM K_2SO_4. When present: 0.2 mM vanadate (V), 5 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). Ethanol was added at 0.17 M final concentration.

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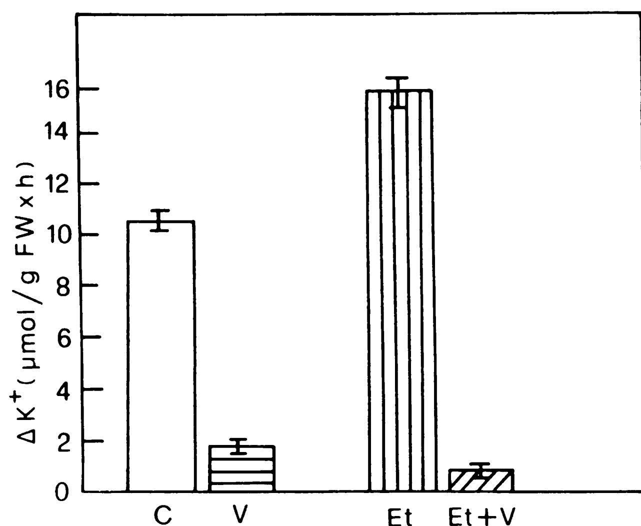

Figure 5. Ethanol-induced changes in net K^+ influx. Control solution (C): 10 mM Mes/BTP, pH 5.5, 0.5 mM $CaSO_4$, 5 μM DCMU, 0.25 mM K_2SO_4. When present, 0.2 mM vanadate (V). Ethanol (Et) was added at 0.17 M final concentration. Bars represent ± SE.

date. This ethanol-induced hyperpolarization occurred both in the absence and in the presence of K^+ in the external medium (Fig. 4, a and b), a behavior repeating the one previously reported for the effects of FC and light (14). Thus, the presence of K^+ in the medium is also required for the effect of ethanol on H^+ extrusion but not for that on E_m. The simplest interpretation (in this case as in those of FC and light activation of H^+ extrusion) is that in the absence of K^+_o, ethanol is still able to stimulate the H^+-ATPase, but the hyperpolarization (consequent to the extrusion of an amount of H^+ too small to be measured) rapidly inhibits the H^+ pump (22).

The data reported in Figure 5 show that the hyperpolarizing effect of 0.17 M ethanol was associated with a significant increase of K^+ uptake, thus repeating, once again, a feature already observed in the cases of the activation of H^+ extrusion by FC and by light (13, 14). Vanadate completely suppressed the stimulation of K^+ uptake by ethanol.

Intracellular pH Changes Induced by Ethanol

The possibility that ethanol might have influenced H^+ extrusion by acidifying the cytoplasm was suggested by previous reports showing that H^+ transport is stimulated, both in vivo and in vitro, by a decrease of cytosolic pH in the pH 8 to 6.5 range (23, 26, 27). The possibility seems to be ruled out by the results of the experiments reported in Figure 6A, showing that 0.17 M ethanol, in the presence of 1 mM K_2SO_4, induced a progressive alkalinization of the cell sap already significant after 15 min of treatment, whereas a much smaller increase from the initial pH was induced by K^+ alone. In the absence of K^+_o, a slight acidification was observed, independent of the presence or absence of ethanol.

Cell sap pH can be considered a measurement of vacuolar pH (16). A more specific, although essentially qualitative, evaluation of the induced changes in "bulk cytoplasmic" pH was obtained by the weak acid distribution method. As shown in Figure 6B, the simultaneous presence of ethanol and K^+ significantly enhanced the rate of DMO uptake and induced a marked increase in the time required for the out-in equilibration of the probe, thus indicating a progressive alkalinization of the cytosol. In the absence of K^+_o, no significant difference in DMO accumulation was found between the ethanol-treated and the untreated samples. A calculation of cytosolic pH (see "Materials and Methods") showed that ethanol and K^+ are synergic in their alkalinizing action, as was also observed for the H^+ extrusion-stimulating action (see Fig. 2).

Changes in Intracellular ATP and Malate Levels

The data of Table I show that 0.17 M ethanol, in the presence of K^+, markedly depressed the ATP level measured

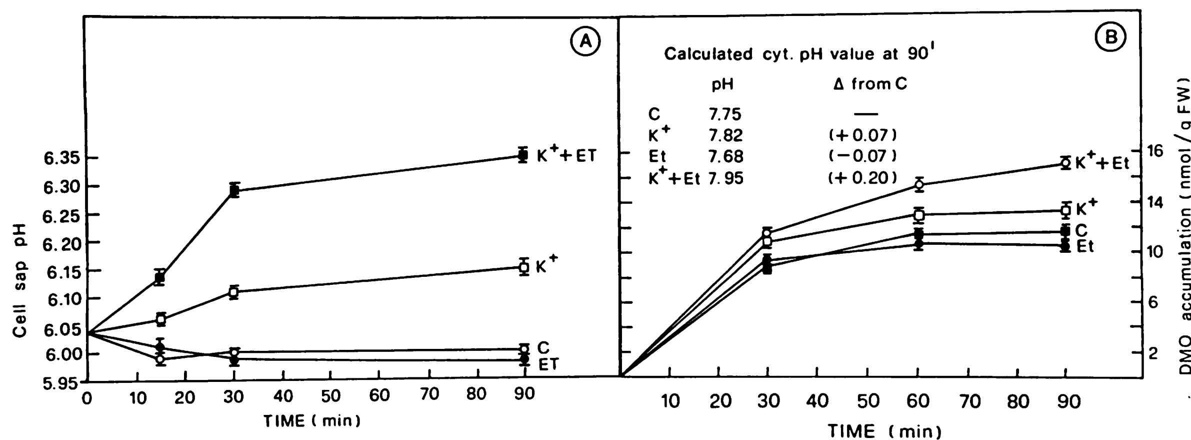

Figure 6. Ethanol-induced changes in cell sap pH (A) and DMO accumulation (B) in the absence and in the presence of 2 mM K^+ in the medium. The cytoplasmic pH values, calculated at 90 min, are reported in the inset. Control medium (C), 10 mM Mes/BTP, pH 5.50, 0.5 mM $CaSO_4$, 5 μM DCMU; K^+, control + 1 mM K_2SO_4. When present, 0.17 M ethanol (Et). DMO was added at 5 μM (155 kBq/μmol) concentration. Bars represent ± SE.

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Table I. *Effect of Ethanol on Intracellular ATP and Malate Levels*

Control: 10 mM Mes/BTP pH 5.50, 0.5 mM $CaSO_4$, 5 μM DCMU, 1 mM K_2SO_4. When present, 0.17 M ethanol and 10^{-4} M FC. Data are means of three replication ± SE

	ATP Content at 60 min	Malate Content at 60 min
	nmol/g fresh wt	*µmol/g fresh wt*
Control ($-K^+$)	140 ± 5	7.76 ± 0.11
+ K_2SO_4	135 ± 1	8.5 ± 0.3
+ Ethanol	158 ± 7	8.61 ± 0.31
+ Ethanol + K^+	110 ± 4	10.5 ± 0.3
+ FC	150 ± 5	
+ FC + K^+	108 ± 1	

in the leaves after 60 min of treatment. This effect was very similar to that induced by FC + K^+, in full agreement with the results previously reported by other authors and interpreted as being due to the increased utilization of ATP by the H^+-extruding ATPase (31). The slight increase of ATP induced by ethanol in the absence of K^+_o (no H^+ extrusion occurring) might be due to the easy utilization of ethanol as a respiratory substrate by *Elodea* leaves (V. Trockner, M. Marrè, unpublished data).

An increase in malate level, associated with the stimulation of H^+ extrusion by treatments with FC, IAA, or light, has been reported in various materials, and it has been interpreted as depending on the stimulation of phospho*enol*pyruvate carboxylation (9, 20, 30). The data of Table I show that also in the case of treatment with 0.17 M ethanol, the stimulation of H^+ extrusion is associated with an increase by 25% of intracellular malate. It is worthy to note that, given the distribution (mainly vacuolar) to malate in the cell, this moderate increase of "bulk" malate concentration might correspond to a much larger percentage increase of this metabolite in the cytosol (25).

DISCUSSION AND CONCLUSION

The results of the present investigation show that in *E. densa* leaves, ethanol markedly stimulates apparent H^+ extrusion, with a maximum effect at the 0.17 M concentration, higher concentrations becoming progressively inhibitory. This effect seems relatively specific for ethanol and propanol. Other alcohols tested are either inactive (methanol, butanol, isopropanol) or inhibitory (pentanol). Among glycolytic metabolites tested, glucose is inactive, whereas glycerol induces some slight stimulation of H^+ extrusion.

In our experimental conditions, the effect of ethanol on apparent H^+ extrusion was strictly dependent on the presence of K^+_o and was associated with (a) an increase in K^+ uptake; (b) a hyperpolarization of E_m; (c) an alkalinization of the cell sap and of the cytosol; (d) an increase in malate level; and (e) a decrease in ATP level. Previous work with FC had shown that all of these responses can be interpreted as being due to a stimulation of the plasmalemma H^+-ATPase (13). The interpretation that ethanol-induced H^+ extrusion is mediated by an activation of the ATP-driven H^+ pump is also supported by its inhibition by the H^+-ATPase inhibitor, vanadate.

The magnitude of the effect of ethanol in promoting H^+ extrusion and associated responses in *Elodea* leaves is similar to those of either FC or light. All available evidence points to the conclusion that the enhancement of H^+ extrusion by FC, light, or ethanol is mediated by an increase in activity of the plasmalemma H^+-ATPase and that it is not a consequence of either a depolarization of E_m or an acidification of the cytosol. Among the experiments reported in this paper, those in which these three stimulating factors have been combined together have shown a partial additivity for ethanol and FC, as well as for light and FC, suggesting that the mode of action of FC is in some way different from that of either ethanol or light. On the contrary, no additivity at all was observed between light and ethanol, suggesting a strict similarity in the mechanisms of action of these two factors.

In the case of both factors, the simplest interpretation of the activation of the pump seems to be that it is mediated by some change in metabolism able to influence the state of activation of the H^+-ATPase in the plasma membrane. In the case of photosynthesis-induced activation, we considered the possibility of a relationship between this effect and the light-induced increase of malate level (7). It is interesting that an increase in malate (presumably via acetylCoA [5]) is also observed when H^+ extrusion in *Elodea* leaves is stimulated by ethanol. Changes in malate level or, more generally, in the metabolic area to which malate belongs, are presumably associated with changes in important regulatory factors, such as, for example, pyridincoenzymes and thiol compounds (for discussion see refs. 17 and 20). Further research is obviously required to throw light on this and other possibilities.

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