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Membrane Potential and Proton Cotransport of Alanine and Phosphate as Affected by Permeant Weak Acids in *Lemna gibba*[1]

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BARBARA BASSO* AND CORNELIA I. ULLRICH-EBERIUS
Dipartimento di Biologia, Università di Milano, I-20133, Italy (B.B.), *and Institut für Botanik, Technische Hochschule Darmstadt, D-6100 Darmstadt, Federal Republic of Germany* (CI.U.-E.)

ABSTRACT

The treatment of *Lemna gibba* plants with the weak acids (trimethylacetic acid and butyric acid), used as tools to decrease intracellular pH, induced a hyperpolarization of membrane potential, dependent on the concentration of the undissociated permeant form of the weak acid and on the value of the resting potential. Measurements were carried out both with 'high potential' and 'low potential' plants and the maximum values af acid induced hyperpolarizations were about 35 and 71 millivolts, respectively. Weak acids influenced also the transient light-dark membrane potential changes, typical for photosynthesizing material, suggesting a dependence of these changes on an acidification of cytoplasm. In the presence of the weak acids, the membrane depolarization induced by the cotransport of alanine and phosphate with protons was reduced; the maximum reduction (about 90%) was obtained with alanine during 2 millimolar trimethylacetic acid perfusion at pH 5. A strong inhibition of the uptake rates (up to 48% for [^{14}C]alanine and 68% for ^{32}P-phosphate) was obtained in the presence of the weak acids, both by decreasing the pH of the medium and by increasing the concentration of the acid. In these experimental conditions, the ATP level and O_2 uptake rates did not change significantly. These results constitute good evidence that H^+/solute cotransport in *Lemna*, already known to be dependent on the electrochemical potential difference for protons, is also strongly regulated by the cytoplasmic pH value.

Uptake of amino acids, sugars, and anions against their chemical or electrochemical gradient has been found to be coupled to H^+ influx, which is energized by the proton electrochemical potential difference $\Delta\tilde{\mu}H^+$ (5, 18). The purpose of the present work was to investigate whether and how cytoplasmic pH influences H^+/solute cotransport in *Lemna gibba*.

It has already been shown that in *Lemna*, apart from the energy requirement, the intracellular solute concentration can become rate limiting for the uptake (24). Phosphate uptake was additionally stimulated with a more negative membrane potential (E_m)[2], though this at the same time implies a higher energy requirement. Therefore it was assumed that the cytosolic H^+ concentration might also have a strong regulating effect on H^+/solute cotransport. There is already some evidence that the uptake has quite a narrow pH_c optimum, for phosphate and glycine uptake in yeast (1, 3), for glucose uptake in *Chlorella* (12), and for Cl^- uptake in *Chara* (20).

To investigate such a regulating effect of pH_c on solute uptake in higher plants, permeant weak acids were employed for manipulating pH_c (1, 2, 4, 9, 10, 15, 17, 19, 22). Weak acids permeate into cells in their undissociated form and they dissociate in the alkaline cytosol. The released protons acidify the cytosol and presumably stimulate the H^+-extruding ATPase at the plasmalemma by acting as a substrate and by shifting pH_c (usually 7.2–7.4 in plant cells (2)) toward the optimum for the ATPase (pH 6.5) (19). This leads to membrane hyperpolarization. Consistent with this, the weak acid-induced effect can be prevented by metabolic inhibitors such as cyanide (10).

It has already been shown for oat coleoptiles that acetic acid at pH 5 severely reduced the amino acid-induced membrane depolarization (9). In the present work the influence of a modulated pH_c on alanine/ and phosphate/H^+ cotransport in *Lemna* is investigated using an electrophysiological approach and determining the concomitant solute-uptake rates, ATP levels and O_2-uptake rates.

MATERIALS AND METHODS

Plant Material. *Lemna gibba* L. G1 plants (obtained from the *Lemna* collection of Professor R. Kandeler, Vienna, Austria) were grown under short-day conditions (8 h light of 30 W m^{-2} at 26°C and 16 h dark at 22°C) in axenic culture in a medium containing 3.96 mM KNO_3, 5.47 mM $CaCl_2$, 1.22 mM $MgSO_4$, 1.47 mM KH_2PO_4, trace elements, and 29 mM sucrose. Plants were pretreated to obtain the two different energy states typical for *Lemna* ('high E_m' and 'low E_m', where high E_m stands for a more negative membrane potential and low E_m stands for a less negative one) previously described (23).

For alanine experiments plants were transferred into a culture medium without sucrose and kept in the dark for 5 or 6 d before the experiments. For phosphate experiments plants were grown without phosphate for 9 d under long-day conditions and for a further 5 d in the dark. The experimental solution (1× [7]) contained 1 mM KCl, 1mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, with or without 0.95 mM NaH_2PO_4, and was adjusted to pH 5 with 5 mM Na-Mes unless otherwise indicated. All experiments were performed in the dark at 25°C.

Membrane Potential Measurements. Plants were fixed in a vertical 4-ml Plexiglas cuvette which was continuously perfused with the experimental solution at a rate of 10 ml min^{-1}. The

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[2] Abbreviations: pH_o, extracellular pH; pH_c, cytosolic pH; $\Delta\tilde{\mu}H^+$, electrochemical potential difference for protons; E_m, transmembrane electrical potential; BA, butyric acid; TMA, trimethylacetic acid.

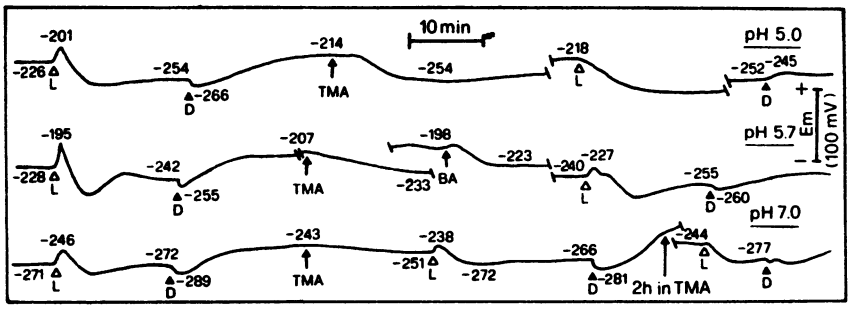

FIG. 1. pH dependence of light/dark-induced transient membrane potential changes and of membrane hyperpolarization as affected by 1 mM TMA or 1 mM BA in *Lemna*. Actual TMAH concentrations: at pH 5, 510 μM; at pH 5.7, 178 μM; at pH 7, 1 μM. Numbers at the traces denote recorded mV. L, light; D, dark. Addition of weak acids indicated by arrows.

Table I. *E_m Hyperpolarization (ΔE_m) by 1 mM Weak Acids in High and Low E_m Lemna, at pH 7, 5.7, and 5*

Number of experiments (*n*).

Plants	pH	Resting Potential E_m	ΔE_m
		mV ± SD	
High E_m	7	−238 ± 20 (11)	6 ± 3 (10)
	5.7	−219 ± 12 (9)	26 ± 4 (4)
	5	−224 ± 15 (27)	35 ± 9 (7)
Low E_m	5	−160 ± 25 (6)	71 ± 5 (5)

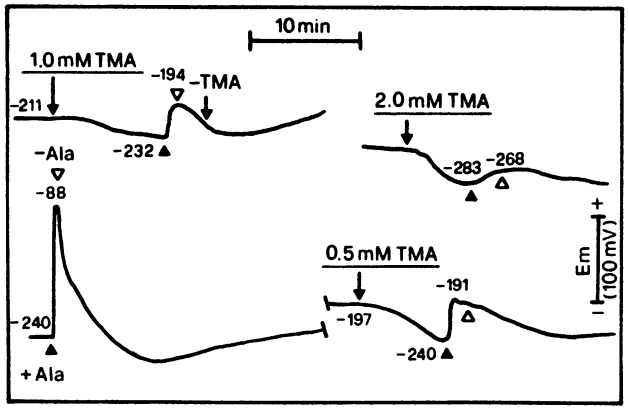

FIG. 2. Alanine-induced membrane potential changes as affected by TMA in *Lemna*; 5 mM alanine, pH 5, dark. Actual TMAH concentrations: at 0.5 mM TMA, 255 μM; at 1.0 mM TMA, 510 μM; at 2.0 mM TMA, 1020 μM. Numbers at the traces denote recorded mV. Closed arrow heads indicate addition and open arrow heads removal of alanine. Addition of TMA indicated by arrows.

glass micropipette, filled with 3 M KCl, and the reference salt bridge (3 M KCl in 1.5% agar) were connected to the electrometer amplifier by Ag/AgCl wires and to a chart recorder.

Microelectrodes were inserted into the plants with a Leitz micromanipulator using a horizontally mounted microscope. Further details were described earlier (16).

The recorded E_m values most probably represent voltage differences only across the plasmalemma, because the same resting potential values were found in parallel experiments (CI Ullrich-Eberius, unpublished data) in which the membrane potential difference was measured associated with the cytosolic pH (7.4) with a pH sensitive microelectrode prepared as recently described by Bertl and Felle (2).

Uptake Experiments. The plants pretreated as described above were transferred into 1× solution with or without phosphate the day before the experiments. Uptake was started by adding L-[^{14}C]alanine or [^{32}P]NaH_2PO_4 (Radiochemical Centre Amersham, United Kingdom). The uptake reaction was stopped by rinsing the plants three times with ice-cold unlabeled experimental solution for 5 min. ^{14}C radioactivity was determined in the dried plants on planchets. The ^{32}P content was measured after hot digestion of the plants with concentrated H_2SO_4, 60% $HClO_4$, and 30% H_2O_2, by counting the Cerenkov radiation in the clear solution.

ATP Determination. *Lemna* plants (150 mg fresh weight) were transferred to 1× solution pH 5 the day before the experiments. Then, as reported earlier (16), they were preincubated for 1 h in the dark by floating on 10 ml 1× solution in 50 ml glass beakers covered by parafilm, before 1 or 2 mM TMA (pH 5) was added. The beakers were gently shaken on a Plexiglas rack in a Warburg apparatus. The fronds were rapidly frozen in liquid N_2 to stop the incubation and they were immediately homogenized with a microdismembrator (Braun). ATP was extracted with cold 5% $HClO_4$ and after neutralization, determined enzymically by the luciferin-luciferase assay in a biocounter (M2010, Abimed).

Respiration. O_2 uptake was measured in the dark manometrically in a Warburg apparatus. Plant material was preincubated as for ATP determination before 1 or 2 mM weak acid was added.

RESULTS

Membrane Potential. The effects of two different weak acids (TMA, pK 5.03 and BA, pK 4.8) were tested on the membrane potential of intact *Lemna* plants. Both these weak acids, at 1 mM concentration, induced a hyperpolarization of similar amplitude (Fig. 1). With decreasing pH_0 from 7 to 5 the hyperpolarization increased from 6 to 35 mV on average in high E_m plants (Fig. 1 and Table I). Plants with an E_m less negative than −180 mV responded with a strong hyperpolarization of 71 mV on average (Table I).

TMA and BA also changed the typical light/dark-induced E_m transients. The first fast membrane depolarization after switching light on and the first fast hyperpolarization after switching light off, as typically occurring in green plant cells, were reduced, and

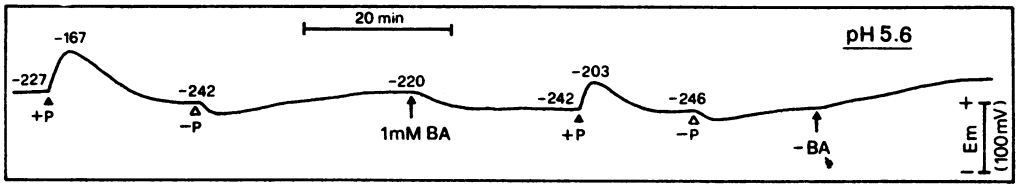

FIG. 3. Phosphate-induced membrane potential changes as affected by 1 mM BA in *Lemna*; 1 mM NaH_2PO_4, pH 5.6, dark. Actual BAH concentration 139 μM. Numbers at the traces denote recorded mV. Closed arrow head indicates addition and open arrow head indicates removal of phosphate. Addition of BA indicated by arrow.

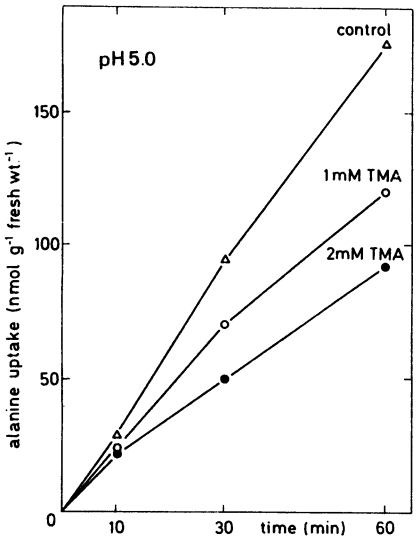

FIG. 4. Time course of [^{14}C]alanine uptake in the absence or presence of TMA in *Lemna*; 0.1 mM alanine, pH 5, dark. Mean values (n = 6).

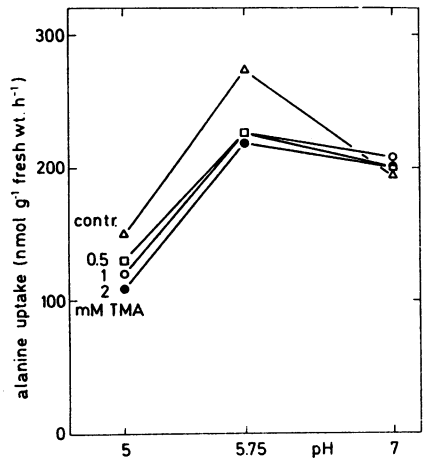

FIG. 5. pH dependence of [^{14}C]alanine uptake as affected by TMA in *Lemna*; 0.1 mM alanine, uptake period 30 min, dark. Mean values (n = 6).

finally completely suppressed by TMA at pH 5 (Fig. 1).

Upon the onset of alanine transport across the plasmalemma of *Lemna* plants, a characteristic E_m depolarization of up to 167 mV occurred, due to H^+ cotransport (8) (Fig. 2). When alanine was applied in the presence of increasing TMA concentrations, there was a distinct and increasing reduction of this depolarization from an average value of 135 ± 15 mV (4 experiments) to a minimum depolarization of 15 mV with 2 mM TMA at pH 5 (Fig. 2).

At the onset of phosphate uptake, E_m transiently depolarized by 60 mV on average (Fig. 3), due to $2H^+/H_2PO_4^-$ cotransport (24). After treating the plants with 1 mM BA at pH 5.6 the depolarization decreased to 65% of the control (Fig. 3).

Alanine and Phosphate Uptake. [^{14}C]Alanine uptake experiments were performed as a function of time and as a function of external pH, in the presence of different concentrations of TMA. Time dependent uptake at pH 5 (Fig. 4) was increasingly inhibited with an increase in TMA concentration. After 1h at 2 mM TMA, the uptake was inhibited by 50%. Alanine uptake, as dependent on external pH (Fig. 5), shows the maximum inhibition by weak acid at pH 5 (30% with 2 mM TMA). At pH 5.75 inhibition was only 20% and no inhibition was detectable at pH 7. The discrepancies between the uptake values at 30 min and at

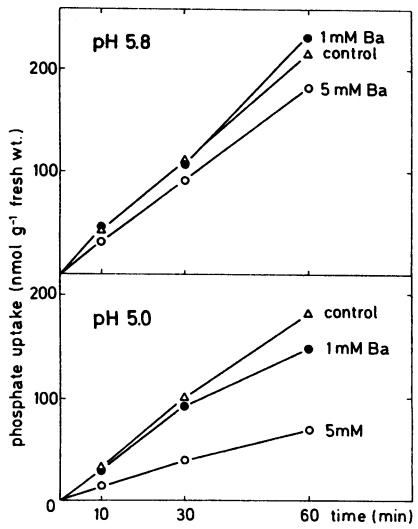

FIG. 6. Time course of ^{32}P-phosphate uptake in the absence or presence of BA at pH 5.8 and pH 5 in *Lemna*; 50 μM NaH_2PO_4, 1 h pretreatment with BA, dark. Actual BAH concentrations: at pH 5.0, at 1 mM BA, 392 μM and at 5 mM BA, 1960 μM; at pH 5.8 at 1 mM BA, 91 μM and at 5 mM BA 455 μM. Mean values (n = 2).

Table II. *ATP Level in Lemna during Incubation with TMA at pH 5*

TMA (1 mM and 2 mM) were tested during two different experiments. Number of experiments = 3.

Concn	ATP Level			
	Control		TMA	
	0 min incubation	60 min incubation	30 min incubation	60 min incubation
mM	*nmol g⁻¹ fresh wt ± SD*			
1	41.5 ± 1.0	40.9 ± 3.6	40.7 ± 1.3	39.3 ± 1.8
2	55.0 ± 8.8	60.3 ± 10.4	65.0 ± 6.6	65.8 ± 4.7

pH 5 reported in Figure 4 and those reported in Figure 5 are to be ascribed to the characteristic energy state (high or low E_m) of the plants.

Also phosphate uptake was inhibited by weak acid: Figure 6 shows that BA induced inhibition of phosphate uptake measured with increasing BA concentration and with decreasing pH_0. The maximum inhibition (70%) was obtained at pH 5 with 5 mM BA (Fig. 6). At pH 5.8, however, 1 mM BA did not inhibit, but even slightly stimulated phosphate uptake.

ATP and Respiration. The influence of 1 and 2 mM TMA at pH 5 on the ATP level of *Lemna* plants was followed for 1 h, *i.e.* for the longest time and highest concentration used for E_m measurements and alanine uptake. During this period no significant change could be detected (Table II).

O_2 uptake during the same period of time and at pH 5 was slightly reduced by TMA, by up to 24% of the control. BA, however, slightly stimulated O_2 uptake by about 14% of the control (Table III).

DISCUSSION

As in green plant cells in general (6, 14), also in duckweed the membrane potential (E_m) shows characteristic transient changes upon switching light on and off (Fig. 1). The data in the figure show that weak acids affect quickly and characteristically the membrane potential of *Lemna* plants. The first rapid depolarization upon switching light on, and the first rapid hyperpolari-

Table III. *O_2 Uptake during Incubation (60 min) with TMA at pH 5 and BA at pH 5.6*

Number of experiments = 3 to 6.

Control	O_2 Uptake		
	1 mM TMA	2 mM TMA	1 mM BA
	nmol g^{-1} fresh wt·h^{-1} ± SD		
4.5 ± 0.9	3.8 ± 0.6	3.4 ± 0.6	
4.5 ± 0.8			5.2 ± 0.5

zation upon switching light off, get smaller and finally disappear after pretreatment of the plants with either weak acid, TMA or BA (Fig. 1). Since these changes are also prevented in the presence of DCMU (23) it is very probable that they are induced by changes in PSII-dependent CO_2 fluxes. Upon illumination, CO_2 from the cytosolic HCO_3^- is consumed by chloroplasts at high rates, thus inducing a transient increase in the OH^- concentration in the cytosol. This could either lead to a transient decrease in H^+-ATPase activity at the plasmalemma or, according to another hypothesis (11), this alkalinization might open K^+ channels, thus leading to an increased, depolarizing K^+ influx. Upon switching light off the cytosolic CO_2 concentration will transiently increase. The CO_2 released by mitochondria will hydrate to H_2CO_3 which dissociates into $HCO_3^- + H^+$, thus causing a transient acidification of the cytosol. This acidification may either directly activate the H^+-extruding ATPase or induce a closure of K^+ channels and hence a transient E_m hyperpolarization. Upon acidifying the cytosol by permeant weak acids these transients were completely suppressed (Fig. 1), supporting our assumption that these first fast light/dark-induced E_m transients originate from changes in the cytosolic CO_2 concentration, and hence from cytosolic pH changes.

Not only were these light/dark transients changed by weak acids but also the membrane potential of *Lemna* measured in the presence of weak acid became more negative with decreasing pH (Figs. 1 and 2), as was also found in several other plant tissues (2, 4, 9, 10, 13, 15, 19). According to the Mitchell hypothesis, the proton pump localized at the plasmalemma gives rise to a proton electrochemical potential difference ($\Delta\tilde{\mu}H^+$) across the membrane, which can be considered as the sum of differences in pH and in electric potential. Neutral and acidic amino acids or anions, which are known to be transported with protons, enter the cell driven by both these favorable differences (1, 8–10, 18). Thus in our experiments the membrane hyperpolarization in *Lemna* by weak acids should increase the rate of alanine and phosphate/H^+ cotransport, as found for K^+ influx (15, 19, 22). On the other hand, weak acids have been shown to decrease pH_c (1, 2, 4, 17) and thus to decrease the H^+-concentration gradient across the plasmalemma. This should result in a decrease of H^+/solute cotransport. The finding that treatment with weak acids did, indeed, inhibit alanine and phosphate uptake seems to indicate that, under our experimental conditions, the H^+/solute cotransport depends on the pH difference across the plasmalemma rather than on the membrane potential. The possibility of an impairment of cellular metabolism, as caused by a toxic side effect of the weak acids, was shown to be quite improbable in *Lemna* plants by ATP and respiration measurements carried out under the same experimental conditions (Table II and III). However, the possibility of weak acid-induced changes of membrane properties influencing aminoacid transport cannot be excluded. Earlier BA was found to be metabolized by only about 2% and TMA was assumed to be metabolized even less (19). Consequently in our experiments we preferentially applied TMA.

From mere E_m measurements it cannot be decided whether a reduction of the H^+/solute-induced membrane depolarization (ΔE_m) is due to an enhanced H^+-ATPase responsiveness as was found under light conditions (16) or due to inhibited H^+/solute influx. Our phosphate uptake experiments showed that at pH 5.8 and 1 mM BA (actual BAH concentration 91 μM) uptake was slightly increased (Fig. 6), whereas the concomitant membrane depolarization was clearly reduced (Fig. 3). Hence this smaller ΔE_m results from an enhanced responsiveness of the H^+ATPase at the plasmalemma. This would also explain the apparently strong reduction of the alanine-induced ΔE_m (Fig. 2). It results from both a weak acid-induced inhibition of H^+/alanine uptake and a weak acid-stimulated E_m hyperpolarization.

A decrease in cytosolic pH might influence the H^+/solute cotransport in two ways: (a) thermodynamically, by decreasing the ΔpH across the plasmalemma and (b) kinetically, in as much as changes in cytosolic pH might influence the characteristics, and thus the activity of the presumably proteic system involved in aminoacid transport. The cytosolic pH change required to energetically counterbalance the E_m hyperpolarization of 35 mV on average in high E_m *Lemna* (Table I) would be about 0.6 pH units.

Thus, the cytosolic pH should decrease from 7.4 to 6.8. In low E_m *Lemna*, a hyperpolarization upon treatment with 1 mM weak acids of up to 71 mV on average (Table I) would be compensated by a pH decrease of 1.2 units. These values are consistently higher than the weak acid-induced pH decrease measured in other plant cells. The pH decreases are: in oat coleoptiles 0.48 (4), in *Sinapis* root hairs 0.21 (2), in barley root tips 0.3 (17). This might suggest that the weak acid-induced inhibition of H^+/solute cotransport in *Lemna* is not the consequence of a decrease in ΔpH, but must involve pH-dependent changes in the kinetic activity of the transporting system. A rather strong dependence of transport on pH_c has already been found in lower plants, such as *Chlorella* (12), the yeast *Saccharomyces cerevisiae* (1, 3) and *Chara* (20).

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