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Biochimica et Biophysica Acta 1285 (1996) 175–182

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

H^+/K^+ exchange in reconstituted yeast plasma membrane vesicles

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Received 21 March 1996; revised 18 June 1996; accepted 10 July 1996

Abstract

The activity of a putative H^+/K^+ exchange system in the plasma membrane of yeast was studied following the alkalinization of the interior of vesicles prepared with lecithin and yeast plasma membrane containing pyranine entrapped inside. The fluorescence of pyranine was used as an indicator of the internal pH of the vesicles. The addition of monovalent cations produced an increase of the internal pH, probably due to the activity of an exchange system, allowing H^+ to leave the vesicle in an exchange for the cation added. The system showed partial selectivity towards K^+ against other monovalent cations, and it was inhibited by amiloride. The activity of this system required the presence of the yeast plasma membrane in the vesicles, and it did not produce important changes of the membrane potential of the vesicles. The exchange depended partially on the relative values of the internal and the external pH of the vesicles. The system shows low affinity for the cations, and appears to be different from the mitochondrial H^+/K^+ exchange system, which is non-selective toward the different monovalent cations. This system could be involved in the regulation of the internal pH of the cells when they accumulate high concentrations of K^+.

Keywords: Ion transport; H^+/K^+ exchange; Plasma membrane; *Saccharomyces cerevisiae*

1. Introduction

Yeast cells are able to transport K^+; this process is driven by a membrane potential difference and a pH gradient generated by a H^+-ATPase, which permit the entrance of K^+ into the cells. [15–17]. This mechanism is common to other yeasts, fungi and even plants [6], and in yeast it is specific for K^+ [1].

Certain peculiarities of this system make it an interesting object of study: first, as was found by Rothstein and Demis [20], the addition of K^+ ions to the incubation medium of yeast stimulates fermentation. Peña et al. [16] found that it also stimulates respiration; this is due to the energy required for the transport of the cation [16,17]. Then, it would be expected that if one measures simultaneously the uptake of K^+ and the rate of fermentation, once the net influx of the cation has stopped, the stimulation of fermentation should also stop; however, this was shown not to be the case; after the uptake or net

Abbreviations: DiSC$_3$(3), dithiacarbocyanine; MES, ((2-[N-morpholino]ethanesulfonic acid); Hepes, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) ; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid) 1,4-piperazinediethanosulfonic acid; PMSF, phenylmethylsulfonyl fluoride; Tris, Tris hydroxymethylaminomethane; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

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PII S0005-2736(96)00153-8

transport of the cation has stopped, fermentation continues to be accelerated [16].

Rothstein and Bruce [19] also showed that, under similar conditions, in yeast, once the net transport of potassium ions has stopped, if $^{42}K^+$ was added, a clear net entrance could be demonstrated, which they interpreted as due to the presence of an exchange system for potassium operating when the cells have attained a concentration equilibrium between internal and external K^+.

It was also shown that, in phosphatidylcholine-yeast membrane vesicles, the addition of ATP, which drives the accumulation of protons into the vesicles, can produce the uptake of ^{86}Rb, apparently against an electrochemical potential difference; this finding can also be explained by the existence of an H^+/K^+ exchange system [18]. With these antecedents, it was considered important to investigate the existence of such exchange system in the yeast plasma membrane.

2. Materials and methods

2.1. Cells

Saccharomyces cerevisiae, (strain R 757, kindly donated by Dr. R. Gaber) was grown in YPD medium (2% glucose, 1% yeast extract, 1% gelatin peptone) for 20 h (to the diauxic phase) at 30°C under continuous shaking. The cells were collected by centrifugation and washed twice with distilled water.

2.2. Mitochondria and plasma membrane preparation.

100 g of yeast (wet weight), were resuspended in 100 ml of 1.2 M sorbitol, 50 mM NaH_2PO_4, 1 mM EDTA, adjusted to pH 7.0 with NaOH; 0.001% β-mercaptoethanol was added, and the cells were incubated for 10 min at 30°C. Lyticase (2000 U per gram of cells) was added, and the incubation was continued for 60 min at the same temperature. Afterwards, the suspension was chilled on ice, and all subsequent steps were carried out at 4°C. The suspension was centrifuged at 3000 RPM in an SS34 rotor for 5 min. The pellet was resuspended in sorbitol buffer plus 1 mM PMSF as a protease inhibitor, and sonicated with four cycles of 15 s with 15-s intervals, at 40 watts, in a Branson 250 sonifier. The homogenate was centrifuged at 5500 RPM in a Sorvall SS34 rotor for 10 min. The supernatant was centrifuged again at 12000 RPM for 30 min and the precipitate, which contained the mitochondrial fraction, was saved. The supernatant was centrifuged again at 18000 RPM during 45 min, and the supernatant was discarded. The pellet obtained was resuspended in 1 mM EGTA adjusted with Tris base to pH 7.2, and centrifuged at 18000 RPM for 45 min; the pellet obtained was resuspended again in EGTA-Tris buffer at a concentration of 10 mg of protein/ml, and was stored in aliquots of 1 mg of protein at minus 70°C. The protein concentration was measured with a modification of the Lowry method [12]. Contamination of the plasma membrane preparation was estimated by the measurement of mitochondrial and plasma membrane ATPase activities at different pH values and in the presence of selective inhibitors. ATPase activity was measured by incubating membranes (10 μg) for 10 min at 30° in 10 mM PIPES (pH 6.7 or 8.2), 5 mM sodium azide, 5 mM $MgCl_2$, and 5 mM NaATP, or at pH 8.2 without azide. Mitochondrial ATPase activity is obtained as that sensitive to azide at pH 8.2, and plasma membrane ATPase as that activity obtained at pH 6.7 in the presence of azide. In none of the preparations of plasma membrane obtained, more that 7% contamination by mitochondrial ATPase was observed.

2.3. Reconstitution of plasma or mitochondrial membrane vesicles

The plasma or mitochondrial membranes were reconstituted in phosphatidylcholine vesicles containing entrapped pyranine (8, hydroxy-1-3-6 pyrenetrisulfonic acid), a pH-sensitive fluorescent probe [5,10]. To this purpose, 1 mg (0.1 ml) of plasma membrane or mitochondrial protein was mixed with 10 mg of soybean lecithin (Sigma) previously washed with acetone and ether [9] in 0.86 ml of 20 mM Hepes-KOH buffer (pH 7.0), and 2 mM pyranine (0.04 ml of a 50 mM solution). The mixture was sonified for 2 min at 20 watts, with intermittent pulses (10%). The resulting suspension was passed through a column of BioGel P-10 [2]. The initial fractions, containing the bright yellow vesicles were collected together, diluted 5 times with the same buffer, and centrifuged at

100 000 × g for 35 min at 4°C. The pellet was resuspended in 0.2 ml of 20 mM Hepes-KOH buffer (pH 7.0). In some experiments, liposomes were prepared without the yeast plasma membrane, following this same procedure. The orientation of the membranes in the plasma membrane vesicles was estimated by measuring the ATPase activity as follows: 10 μg of vesicle protein was incubated for 10 min at 30° in 10 mM PIPES (pH 6.7), 1 μM CCCP; 5 mM $MgCl_2$, and 5 mM NaATP, with or without 0.5% Triton X-100. In this way, an inside-out orientation of the membrane proteins of 70% was estimated.

2.4. Fluorescence measurements and estimation of the internal pH

Measurements were carried out in a DMX-1000 SLM spectrofluorometer (Urbana, IL), with a thermostated chamber at 30°C, using 1.0 cm light path glass cells. For each tracing, 2.0 ml of 20 mM Hepes buffer, adjusted to pH 7.0 with KOH, were mixed with 20 μl of reconstituted vesicles (approx. 100 μg of protein). After 1 min, the different cations were added. Changes in pyranine fluorescence were recorded at 460-520 nm for excitation and emission, respectively. A small amount of K^+ – in the buffer from the beginning – was used, since this was found to be convenient to obtain better tracings. Since, besides, the system shows low affinity, it is possible that some basal concentration of KCl may not significantly affect the results and conclusions.

It was verified that the fluorescence changes indicated the values of the internal pH of the vesicles; to this purpose, the vesicles were centrifuged at varying times after its preparation, to measure the concentration of pyranine in the supernatant. It was found that after 2 h, only approx. 10% of the fluorescent dye appeared in the supernatant. In order to calibrate the signal to obtain the actual pH value inside the vesicles, a high concentration of NH_4OH (100 mM) or propionic acid (100 mM) was added to obtain the maximal and the minimal fluorescence values, respectively. After the addition of propionic acid, a small value of the fluorescence remained, probably due to the binding of the indicator to the vesicles. This small value was subtracted from all the values of fluorescence obtained. Then, the corrected values for the maximal fluorescence obtained after the addition of NH_4OH were used as equivalent to the maximal dissociation of the dye; with the corrected value of the fluorescence obtained in every experimental point, made equivalent to the dissociated form of the molecule, and a value of 7.2 for the pK of the dye [10], the actual values for the internal pH were calculated.

2.5. Membrane potential estimation

To estimate the membrane potential, 2.0 ml of 20 mM Hepes-KOH buffer (pH 7.0) were mixed with 20 μl of vesicles reconstituted without pyranine, in a 1.0-cm light path glass cell of the spectrofluorometer. The same conditions as in the fluorescence measurements were used, but the excitation and emission wavelengths were 540-590, respectively. The tracings were started by the addition of 0.125 μM dithiacarbocyanine ($DiSC_3(3)$, Molecular Probes), and then the corresponding cation was added.

2.6. Buffers

In experiments in which different pH values were used, a mixture of 10 mM MES and 10 mM Hepes was used, and adjusted to the desired pH with KOH.

All reagents were obtained from Sigma Chemical Company. Fluorescent indicators, pyranine and $DiSC_3(3)$ were obtained from Molecular Probes (Eugene, OR, USA)

3. Results

Fig. 1a shows the fluorescence changes observed upon the addition of K^+ to vesicles reconstituted with soybean lecithin proteoliposomes and yeast plasma membranes in which the fluorescent probe pyranine was trapped. The addition of K^+ produced an immediate increase in the fluorescence of pyranine entrapped in the vesicles. The effect was clearly dependent on the concentration of KCl, and at least two phases of the fluorescent changes could be detected, a faster initial change in intensity, followed by a slower component. The addition of Na^+ also produced these changes (Fig. 1b) but they were of lower magnitude at equivalent concentrations of cation added. The same results were observed using K_2SO_4

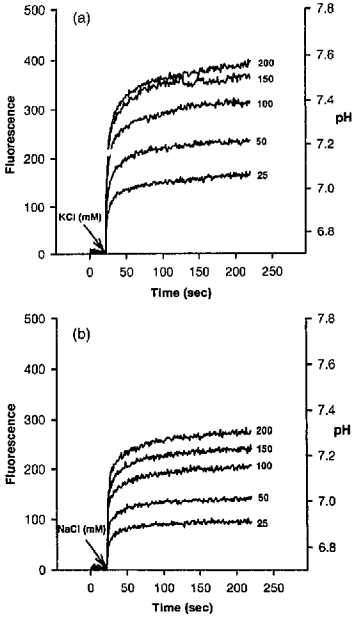

Fig. 1. Changes in fluorescence intensity induced by K^+ or Na^+. Vesicles (20 μl) were preincubated for 1 min in 20 mM Hepes-KOH buffer (pH 7.0), and then different concentrations of KCl (a) or NaCl (b) were added. Pyranine fluorescence was recorded and pH values were calculated as described in Section 2. Temperature was 30°C; final volume was 2.0 ml.

or Na_2SO_4 (not shown). Considering the pH values attained after 200 s, the concentration of KCl necessary to reach the half maximum change in intensity of fluorescence was around 70–100 mM, reflecting a low affinity for K^+. As a control, a similar addition of cations was tested in liposomes prepared in the same way, but without the plasma membrane; NaCl, KCl or even an equal volume of water, instead of an increase, produced a decrease of fluorescence, contrary to liposomes reconstituted in the presence of plasma membrane (not shown). It was also tested that the addition of KCl to a solution of pyranine giving the same fluorescence values as those obtained with the membrane vesicles did not produce significant changes of fluorescence.

The selectivity of the system to different monovalent cations is shown in Fig. 2. There was a selectivity favoring K^+ over Cs^+, Li^+, Na^+ and Rb^+, but first, the selectivity was far from absolute, and second, it favored K^+ over the other monovalent cations. Another test of the selectivity of the system consisted in adding to the reconstituted vesicles either K^+, Li^+, Na^+, Rb^+ or Cs^+, and after 120 s, when fluorescence had reached a plateau, 100 mM K^+ was added. It is clear that K^+ added after any of the other cations still produced a significant change in fluorescence, and also that the lowest fluorescence change was observed if it was added as a second addition of this same cation. To discard effects of surface charge changes, the divalent cations Ca^{2+} or Mg^{2+} up to 0.2 mM, or the trivalent cation Tb^{3+}, up to 40 μM, were added and found to be ineffective in altering the fluorescence changes produced by K^+ (not shown).

Reconstituting by the same procedure the mitochondrial membrane into the vesicles; results were

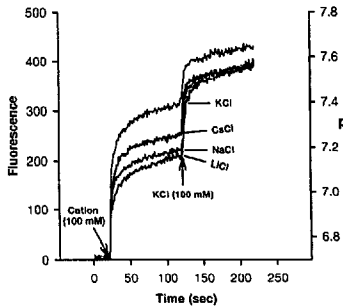

Fig. 2. Selectivity of the H^+/K^+ exchanger. The different monovalent cations were added to reconstituted plasma membrane vesicles. The experiment was performed as in Fig. 1; where indicated by an arrow, at a concentration of 100 mM. Conditions were as for Fig. 1. After 120 s, 100 mM of KCl were added.

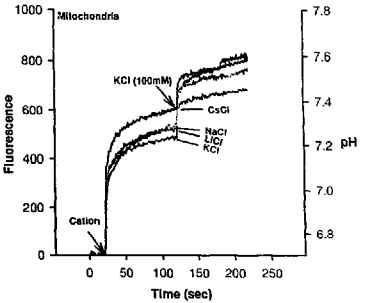

Fig. 3. Changes in fluorescence and calculated values of the internal pH of vesicles prepared with yeast mitochondria with entrapped pyranine. The experiment was carried out as described for Fig. 1, but the vesicles were prepared by the same procedure, using yeast mitochondria instead of the plasma membrane preparation. Yeast mitochondria were prepared as described in Section 2.

similar, but in different experiments, the preparation showed a different selectivity pattern, or no selectivity at all towards the different monovalent cations (Fig. 3).

Cation/H^+ exchange systems have been found to be inhibited by amiloride; in mammalian cells, submicromolar concentrations inhibit Na^+/H^+ exchange [11]. In *Saccharomyces cerevisiae* amiloride has been shown to inhibit acid extrusion and cytoplasmic alkalinization induced by glucose, with an IC50 of 745 μM. This inhibition has been suggested to be due to an inhibition of glucose metabolism [7,8]. In Fig. 4, we show that amiloride could inhibit K^+/H^+ exchange; the inhibition was more effective against potassium than against sodium, but rather high concentrations (400 μM) were required to inhibit the fluorescence changes produced by both cations (Fig. 4a,b).

The fluorescent dye cyanine $DiSC_3(3)$, has been found to respond to the membrane potential changes of the plasma membrane vesicles of yeast, energized by the H^+ pumping activity of cytochrome oxidase [4]. In experiments performed to detect possible changes of the membrane potential, it was found that the addition of both cations did not produce any variations in the fluorescence of the cyanine, indicating that probably these cations are involved in an electroneutral exchange, affecting substantially the internal pH, but not the membrane potential (Fig. 5).

If a K^+/H^+, or other cation exchange system exists, it would be expected to be sensitive to the changes of both internal or the external pH. Varying the internal and the external pH values, (Fig. 6a–c)

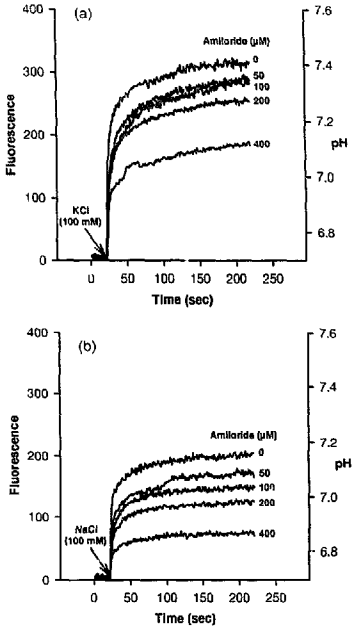

Fig. 4. Inhibition of H^+/K^+ exchange by amiloride. Plasma membrane reconstituted vesicles were preincubated for 5 min in 20 mM Hepes-KOH buffer (pH 7.0), with different concentrations of amiloride. Then 100 mM KCl (a) or NaCl (b) was added.

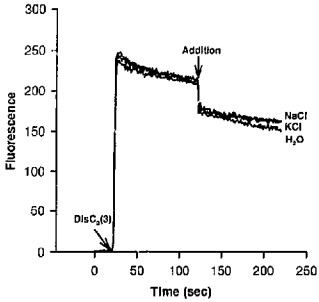

Fig. 5. Estimation of the membrane potential in reconstituted vesicles. 0.125 μM $DiSC_3(3)$ was added to vesicles incubated in 2.0 ml of 20 mM Hepes-KOH. After 1 min, 100 mM KCl, NaCl, or an equivalent volume of water were added. Fluorescence changes were recorded at 540–590 nm.

produced some changes in the response of the fluorescence produced by the addition of KCl. However, it appears that the highest values of the fluorescence changes were always obtained when the external pH was 7.0. Besides, the highest value for the fluorescence change was obtained when the internal pH was 6.0 or 6.5.

Octylguanidine, another substance which was found to inhibit K^+ transport in yeast [14] was also tested; at concentrations of 20 or 40 μM, it was found that its addition produced some increase of the internal pH of the cells; however, it did not modify the response to the further addition of either K^+ or Na^+ (not shown). In other yeasts, the appearance of a Na^+/H^+ exchange system seems to be related to osmotic stress [13,21], so we tested the possibility that growing the cells in high concentrations of NaCl (0.5–1.0 M) would modify the selectivity patterns observed, we did not obtain a significant change in

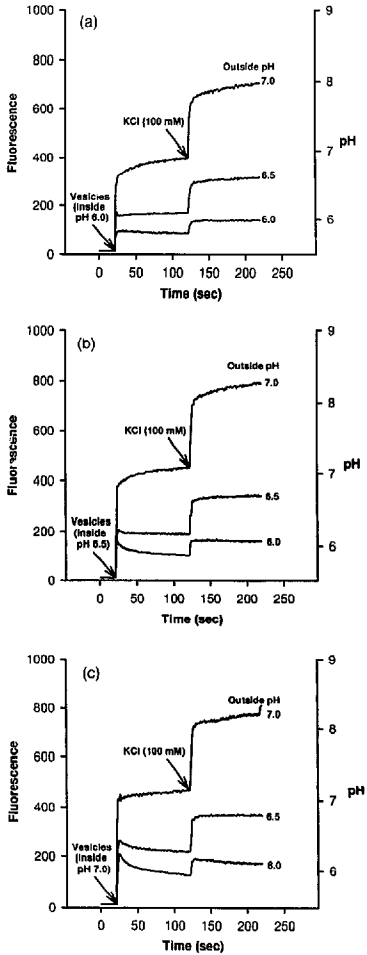

Fig. 6. H^+/K^+ exchange at different internal and external pH values. Vesicles were reconstituted using buffer at pH 6.0 (a), 6.5 (b) or 7.0 (c), and then 20 μl were mixed with 2.0 ml of different pH buffers. After 120 s, 100 mM KCl was added.

the previous results that could confirm our assumption; further work will be required to test this idea.

It was also verified that in this strain, R757, as in the strain used before by Peña et al. [16], continued stimulation of fermentation was observed after the net uptake of KCl had stopped (not shown).

4. Discussion

Our results are consistent with the presence of a cation/H^+ exchange system, with some selectivity toward K^+, which could be demonstrated by the changes of fluorescence of pyranine trapped by the yeast plasma membrane vesicles, a fluorescent dye responding to pH changes by increasing its fluorescence at the used wavelengths; this was indicated by the following findings: (a) the response was observed by the addition of monovalent cations; (b) it depended on the presence of the plasma membrane; liposomes without the plasma membrane showed a completely different response. (c) a relative specificity was found for K^+, as compared to other monovalent cations, although in the preparation used, this specificity was relatively small, and (d) the estimation of the membrane potential changes by means of a fluorescent cyanine showed no changes upon the addition of the monovalent cations. This last characteristic of the system would appear to eliminate the possibility of a simple potassium transport system, which could produce a membrane potential change which could finally constitute a driving force to expel protons from the vesicles through some proton conducting system in the membrane.

It is also interesting that when mitochondrial membranes were used instead of the plasma membrane preparation, similar effects were observed; however, with this preparation, no selectivity was observed, as has already been reported by Welihinda et al. [22]. In any case, by the assay of marker enzymes (mitochondrial vs plasma membrane ATPase activity), a maximum contamination by mitochondrial membranes of about 7% could be observed. On the other hand, the measurement of this same enzyme activity in the presence of an uncoupler (1 μM CCCP), compared to the activity with the further addition of 0.5% Triton X-100 indicated an outward orientation of the plasma membrane enzymes of approx. 70%. In any case, the addition of the monovalent cations was always from the outside; because of this, it would be expected that the activity measured in the system, particularly during the first seconds after the addition of the cation, were that of the assumed carriers with their cation site oriented toward the outside of the vesicles.

The presence of either divalent or trivalent cations did not affect the response of the vesicles to the addition of the monovalent ones. The concentrations used were, of course, much smaller than those for the monovalent cations; however, these concentrations were used, since they are those which either affect significantly the surface charge of the cells, or are transported into them [3].

The affinity toward monovalent cations was low, responding to concentrations in the millimolar range. Although this might appear against the existence of a putative exchange system, such a system could be imagined functioning when the internal pH of the cells and the internal K^+ concentration reached high levels. It would be expected to function with a very low affinity for the monovalent cations, particularly K^+; otherwise, it would represent an inconvenient futile cycle for the cell, short-circuiting the pumping of protons and the uptake of K^+. It is important to point out also that, in spite of the fact that only a relative specificity was observed for the monovalent cations, the experimental system used is not a simple one, and other mechanisms of ion transport may be present in the preparation. About the possible role of such a system in yeast, it may function to regulate the internal pH of the cells; potassium ions indirectly produce an increased pumping of protons, and in doing so they increase the internal pH of the cell. This exchange system could represent a safety mechanism to avoid an excessive alkalinization of the cell interior.

One additional property of the system described is its sensitivity to the diuretic drug amiloride, which was also found more effective against K^+ than against Na^+; however, the inhibition was found at rather high concentrations of amiloride; this drug has a guanido group in its structure, and guanidines have been shown to affect the transport of monovalent cations in yeast [14]. On the other hand, although octylguanidine, one such active substance in inhibiting the transport of K^+ in yeast, produced itself an

increase of the fluorescence of pyranine, it did not modify the response to the further addition of K^+.

When the internal or the external pH of the vesicles was varied, the maximum change in fluorescence intensity was observed when the internal pH was low (6.0 or 6.5), against an external value of 7.0. These results indicate that a pH gradient, acid in the trans side of the K^+ addition, favors the exchange and also that the exchanger might be modulated both by protons and the concentration of K^+ at opposite sides of the membrane. It has to be mentioned that, at low external pH values, a slight acidification of the vesicles was observed before the addition of the monovalent cation; this is due probably to the fact that the vesicles are not 100% sealed to proton movements. This was in fact confirmed by observing the changes of the pyranine fluorescence upon the addition of either HCl or NaOH to the vesicle suspension. Both additions produced either the acidification or alkalinization of the vesicle interior, respectively.

The results agree with other data, starting with those of Rothstein and Bruce [19], who found that when potassium is added to yeast cells, a high rate of labeled potassium uptake is measured after net uptake of the cation has ceased. K^+ in the incubation medium of yeast cells stimulates fermentation, but this stimulation of fermentation by K^+ also persists after the net uptake has stopped [16]. The data also agree with the results in which an influx of $^{86}Rb^+$ was observed in yeast plasma membrane vesicles in spite of the existence of a positive and acid internal medium which was generated by the activity of the H^+-ATPase of the membrane [18].

Acknowledgements

This work was partially supported by grants, IN-202193 from the Dirección General de Asuntos del Personal Académico, of this University, and 3188N from the Consejo Nacional de Ciencia y Tecnología, de México. M.M.-L. is recipient of a Conselho Nacional de Ciencia e Tecnologia (CNPq) fellowship.

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