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Electrochemical potential and ion transport in vesicles of yeast plasma membrane

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Vesicles from yeast plasma membrane were prepared according to Franzusoff and Cirillo ((1983) J. Biol. Chem. 258, 3608), with slight modifications. When Mg-ATP was added, this preparation was able to generate a membrane potential, that was sensitive to inhibitors of the yeast H^+-ATPase and uncouplers, and could be decreased by the addition of permeant anions, as measured by the fluorescence changes of the dye oxonol V. The addition of ATP could also generate a pH gradient, detectable by the fluorescence changes of the monitor aminochloromethoxyacridine. This gradient was sensitive to inhibitors of ATPase and uncouplers, and could be increased by the addition of permeant anions to the incubation mixture. When the vesicles were loaded with KCl, an increased rate of K^+ efflux was produced upon the addition of ATP. Cytochrome oxidase from bovine heart could be reconstituted into the vesicles and was shown to generate a membrane potential difference, negative inside, evidenced by the fluorescence quenching of the cyanide dipropylthiacarbocyanine and the uptake of tetraphenylphosphonium. Besides, in these vesicles, K^+ and Rb^+, but not Na^+ or NH_4^+ could decrease the quenching of fluorescence and the uptake of tetraphenylphosphonium produced when the electron-donor system was present. In the vesicles in which cytochrome oxidase was incorporated, upon the addition of cytochrome *c* and ascorbate, the uptake of $^{86}Rb^+$ could be demonstrated also. This uptake was found to be saturable and inhibited by K^+, and to a lesser degree by Na^+. The results obtained indicate that these vesicles are reasonably sealed and capable of generating and maintaining a membrane potential. The membrane potential could be used to drive ions across the membrane of the vesicles, indicating the presence and functionality of the monovalent cation carrier. The vesicles, in general terms seem to be suitable for studying transport of ions and metabolites in yeast.

Introduction

The plasma membrane of yeast has a H^+-ATPase that generates an electrochemical potential gradient which drives different transport systems [1]. This ATPase has been characterized and isolated from several species of yeast [2–5], and from *Neurospora crassa* [6]. In all kinds of fungi, the general characteristics of the enzyme are simi-

Abbreviations: Mes, 4-morpholineethanesulfonic acid; 1799, a 2:1 adduct of hexafluoroacetone and acetone; DCCD, dicyclohexylcarbodiimide; EDTA, ethylenediamine tetraacetic acid; FCCP, carbonylcyanide trifluoromethoxyphenylhydrazone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; PC, phosphatidylcholine.

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lar [7], with a molecular mass of 105 000 kDa, and an optimal pH around 6.0, differently to the mitochondrial ATPase of these microorganisms, which has an optimum pH around 9.0. Besides, it is inhibited by diethylstilbestrol and vanadate, and not by oligomycin and azide. Both ATPases are inhibited by DCCD and Dio 9 [2,7]. The gene of this ATPase has been cloned and sequenced [8].

It has been possible to reconstitute the isolated plasma membrane ATPase of yeast and fungi into liposomes [9–12]. By this procedure, or by using inside-out vesicles from *Neurospora crassa* [13,14], it was possible to show that the enzyme works as a proton pump capable of generating an electrochemical potential gradient, that has been postulated as responsible for driving the transport of several metabolites, such as Ca^{2+}, K^+, amino acids and sugars [15–19].

With several microorganisms and other biological systems, the use of membrane vesicles has been also useful in the characterization of solute transport. In the fungus *Neurospora crassa*, its plasma membrane can be vesiculated by rather simple procedures [14,20]. With yeast , and especially in the case of *Saccharomyces cerevisiae*, it had been difficult to obtain a preparation of sealed plasma membrane vesicles. These preparations [21], when tested in our laboratory, gave low ATPase activities and were not sealed (unpublished results). However, Franzusoff and Cirillo [22], by a modification of the procedure of Fuhrmann et al. [21], obtained plasma membranes from *Saccharomyces cerevisiae* and prepared from them vesicles that could be used to study the transport of sugars. Another preparation reported recently by Ahlers [23], appears to be sealed and might be useful also for studies on solute transport.

Since the study of Matsushita et al. [24], a bacterial cytochrome oxidase could be incorporated into vesicles of *Escherichia coli*, to drive the transport of lactose by generating a membrane potential. Recently, Driessen et al. [25] and Hirata et al. [26], achieved similar results with vesicles of *Streptococcus cremoris*, and a thermophilic bacterium, respectively, and could demonstrate the uptake of amino acids, driven by the electrochemical potential generated by cytochrome *c* oxidase.

In this report, results are presented of an attempt to determine the ability of vesicles prepared by the procedure of Franzusoff and Cirillo [22], to generate a membrane potential by different means, and that of transporting ions.

Materials and Methods

Cells. Saccharomyces cerevisiae was obtained commercially (La Azteca, S.A.). The cells (30 g of packed cells) were incubated for 8 h at 30 °C under vigorous aeration in 1 l of the medium described by De Kloet et al. [27], placed in a 2 l Erlenmeyer flask. After this incubation, the cells were centrifuged and washed twice by centrifugation with deionized water, and resuspended in 400 ml of water. They were then aerated overnight (15 to 18 h) by bubbling air through the suspension in a glass cylinder. In the morning, the cells were centrifuged and washed once with water.

Preparation of plasma membranes. They were obtained according to the procedure of Franzusoff and Cirillo [22], with an additional resuspension of the membranes in buffer B (10 mM Tris, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3, pH 7.5) at the end of the procedure, and centrifugation at 140 000 × *g* during 40 min, resuspending them finally at a ratio of 10 to 20 mg of protein per ml of the same buffer. The additional washing was in order to eliminate the pyrohosphatase and phosphatase activities found.

Preparation of the plasma membrane vesicles. The procedure of Franzusoff and Cirillo [22] was followed with minor modifications. Liposomes of acetone-washed phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) were prepared by sonication (Branson B-12 sonicator) until the solution was clear, usually for 40 s at a concentration of 47.6 mg per ml in 10 mM Mes-triethanolamine buffer (pH 6.5), 1 mM EDTA, that contained besides either 20 mM sucrose, or 10 mM KCl. Then, 31.5 mg of liposome lipid was mixed with 4 mg of protein, to a final volume of 1.0 ml of the same medium used to prepare the liposomes. The mixture was stirred and twice frozen in an acetone bath cooled with solid CO_2, and thawed afterwards at room temperature. Then the mixture was sonicated in a bath sonicator (Bransonic 32) for variable times, usually 10 min, unless specified otherwise, until the mixture clarified. When cyto-

chrome oxidase was used, the same procedure was used, but the medium was changed to 100 mM phosphoric acid, neutralized to pH 7.0 with triethanolamine.

Preparation and incorporation into the vesicles of cytochrome oxidase from bovine heart. The methods used were described elsewhere [28]. Reconstitution was achieved by mixing the enzyme preparation with the vesicles.

Enzymatic assays. The ATPase activity was measured in 350 μl of a reaction medium containing 10 mM Mes-triethanolamine buffer (pH 6.5), 10 mM $MgSO_4$, 6 mM ATP, 1 mM EDTA and 20 mM sucrose. The amount of protein was 100 μg. The reaction was started by the addition of ATP and the mixture was incubated for 10 min at 30 °C. The reaction was stopped by the addition of 100 μl of 30 percent trichloroacetic acid and the tubes were transferred to an ice bath. They were centrifuged, and hydrolyzed phosphate was measured by the method of Fiske and SubbaRow [29].

Use of fluorescent probes. The generation of a membrane potential and of a pH gradient were followed by the quenching of the fluorescence of oxonol V [30,31] or dipropylthiacarbocyanine and 9-amino-6-chloro-2-methoxyacridine [9,10,23], resp. The vesicles (200 μg protein/ml) were added to a cuvette containing a buffer, and either 20 mM sucrose, 10 mM KCl, or 10 mM potassium acetate. To follow the internally positive membrane potential generated by ATP, 5 mM $MgSO_4$ and 2 μM oxonol V were included, and the reaction was started by the addition of 5 mM ATP. The wavelengths used to measure fluorescence were 580–640 nm. In the vesicles to which cytochrome oxidase was incorporated, the membrane potential, expected negative inside, was followed by the fluorescence quenching of dipropylthiacarbocyanine [32]. The vesicles were added to the incubation medium containing the cyanine, followed by cytochrome *c*, ascorbate, and tetramethyl-*p*-phenylenediamine. The fluorescence of the cyanine was followed at 540–580 nm. To follow the pH changes, the mixture contained 1.5 mM $MgSO_4$ and 1.5 μM aminochloromethoxyacridine; the reaction was started by the addition of 1.5 mM ATP. The wavelengths for excitation and emission were 400 and 440 nm, respectively.

Measurement of ^{3}H-tetraphenylphosphonium uptake. The vesicles (7.5 μg protein) in a final volume of 500 μl were incubated for 5 min with ^{3}H-tetraphenylphosphonium. After incubation, they were passed through a Sephadex G-50 fine column, as described for the measurement of ion uptake (see below). The radioactivity of the vesicles was then measured in a scintillation counter.

Measurement of ion transport. To measure K^+ efflux, vesicles were prepared in a medium with 50 mM KCl, and passed through a column of 22 × 0.6 cm of Sephadex G-50 fine, to eliminate external potassium. Then they were placed in a K^+-free medium, and the efflux of the cation was followed by means of a K^+ electrode (Orion), connected to a pH meter and a recorder. To measure the influx, the vesicles were incubated as indicated under individual experiments in the presence of $^{86}Rb^+$ and, at the indicated times, samples were taken and placed on top of an insulin syringe filled with 1 ml of a suspension of Sephadex G-50, fine, from which excess liquid was previously removed by suction. Then the syringe, placed inside a test tube, was centrifuged for 2 min to 3500 rpm. The volume coming out of the syringe was measured to correct results for dilution, an aliquot was taken and placed in a filter paper to dry. The filter paper was then placed in a scintillation vial with a scintillator and counted.

Protein determination. Protein was measured by the technique of Lowry et al. [33], with bovine serum albumin as standard.

Reagents. Most reagents were obtained from Sigma Chemical Co., St. Louis, MO. Oxonol V and dipropylthiacarbocyanine were obtained from Molecular Probes, Junction City, OR. Sephadex was obtained from Pharmacia Fine Chemicals. Aminochloromethoxyacridine was a kind gift from Dr. P. Overath, Max Planck Institute for Biology, Tubingen, 1799 was kindly provided by Dr. P.G. Heytler, DuPont de Nemours, Wilmington, DE. ^{3}H-Tetraphenylphosphonium was kindly donated by Drs. H.R. Kaback and N. Carrasco, Roche Institute of Molecular Biology, Nutley, NJ.

Results

The plasma membrane obtained by the method of Franzusoff and Cirillo [22] was found to con-

tain, besides the ATPase, substantial phosphatase activity that could be evidenced by using ADP and *p*-nitrophenyl phosphate as substrates, as well as a pyrophosphatase, tested with pyrophosphate as substrate. These activities could be inhibited by molybdate and NaF, respectively, and were eliminated by an additional washing by centrifugation at $140000 \times g$ during 40 min (results are not shown). The ATPase activity of the plasma membrane preparation, measured at pH 6.5, varied between 0.6 and 1.0 $\mu mol \cdot (min^{-1} \cdot mg^{-1})$ (not shown). This activity was similar to that of other reported preparations [7,22,23], and was inhibited between 5 and 10 percent with 10 μg of oligomycin, varying from one preparation to another. Effective inhibitors were vanadate and diethylstilbestrol; triphenyltin and DCCD were partially effective, as found later with vesicles (Table I), even if used at rather high concentrations, or if, as in the case of DCCD, the membranes were preincubated for 20 min with the inhibitor. Azide was ineffective as an inhibitor.

Differently to the report of Franzusoff and Cirillo [22], sonication for 7 s after freezing and thawing twice, did not produce functional vesicles. It was also observed that, with the recommended procedure, the vesicles were not able to generate a potential gradient. This problem, however, seemed to be due to the sonicator used (Branson B32), and could be solved with longer sonication times, in the order of minutes. After sonicating for 3 to 5 min the fusion of the liposomes and the plasma membranes was shown by centrifugation in a Percoll gradient from 1.019 to 1.14 $g \cdot ml^{-1}$ for 45 min at $70000 \times g$ (showing one band, data not shown), and by the ability of the vesicles to form both an electric potential and a pH gradient. It was also tested that sonication of the plasma membranes for up to 15 min, did not produce any decrease of the ATPase activity. Although with rather short sonication times (from 3 to 5 min) it was possible to obtain a satisfactory preparation, but due to the variability of the procedure, it was decided to use 10 min for this sonication step as the usual procedure.

When vesicles were prepared, the ATPase activity varied also from 0.6 to 1.0 $\mu mol \cdot (min^{-1} \cdot mg^{-1})$ (Tables I and II); it was always similar to the activity observed in the membrane preparation. The ATPase activity showed the same sensitivity to inhibitors as the plasma membrane preparations (Table I). This enzyme preparation was also relatively insensitive to DCCD and triphenyltin. One interesting fact, however, is that the ATPase activity of the vesicle preparation was stimulated by lysophosphatidylcholine (Table II), and this stimulation was not observed with the plasma membrane preparation. Besides, a small

TABLE I

ATPase ACTIVITY OF PLASMA MEMBRANE VESICLES FROM YEAST, PREPARED ACCORDING TO FRANZUSOFF AND CIRILLO [22]. EFFECTS OF INHIBITORS

The activity was measured as described under Methods. The inhibitors used were added at the indicated concentrations 2 min before starting the reaction with ATP, except for DCCD, that was preincubated 20 min with the vesicles. Results are the mean of two experiments.

Experimental conditions	Activity	
	specific (nmol $\cdot min^{-1} \cdot mg^{-1}$)	relative (% of control)
Control	936	100
Oligomycin, 10 μg	893	95
100 μM vanadate	92	10
300 μM NaN_3	908	97
500 μM diethylstilbestrol	324	35
500 μM triphenyltin	777	83
143 μM DCCD	797	85
10 μM FCCP	1008	108
Nigericin, 10 μg + 10 mM KCl	1033	110

TABLE II

EFFECTS OF LYSOPHOSPHATIDYLCHOLINE ON THE ATPase ACTIVITY OF PLASMA MEMBRANES AND VESICLES OF *S. cerevisiae*

The experiment was carried out as described under Methods. Lysophosphatidylcholine (lysoPC) was added at a concentration of 0.01 percent.

	Specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Percent activity
Plasma membranes		
Control	836	100
LysoPC	844	101
Vesicles		
Control	796	100
LysoPC	929	117

but consistent stimulation was observed also with FCCP and nigericin in the vesicles (Table I), indicating already some degree of coupling of the ATPase activity to proton pumping, as reported for the reconstituted preparations of the enzymes into liposomes [9–11,34].

Preliminary experiments with the vesicles resulting from the fusion of liposomes and plasma membranes, prepared in 100 mM KCl, showed that, when the vesicles were incubated in a K^+-free medium and valinomycin was added, a diffusion potential, negative inside, was generated and it was sensitive to uncouplers (results not shown), as could be evidenced by the fluorescence quenching of dipropylthiacarbocyanine. ATP added to the incubation mixture was not expected to penetrate into the vesicles, but to be hydrolyzed by those enzymes with their active sites oriented toward the outside of the vesicles. If, as it happens with the isolated enzyme when reconstituted into liposomes [9–11], the ATPase were anisotropically inserted in the vesicles, by hydrolyzing ATP from the outside, it would be expected to pump protons into the vesicles, generating a membrane potential, positive inside, and a pH gradient, acid inside. In this case, it ought to be possible to monitor the membrane potential gradient with a negatively charged probe, like oxonol V [30,31], and the pH gradient with aminochloromethoxyacridine [10,23].

Fig. 1 shows the results of one experiment in which several sonication times were used to obtain the vesicles, following the quenching of aminochloromethoxyacridine upon addition of ATP. The plasma membranes, when mixed with the liposomes, and subsequently frozen and thawed, without sonication, did not produce any quenching of the fluorescence upon addition of ATP. In this particular experiment, it was enough to sonicate for 2 min to obtain maximal activity in the generation, by the addition of ATP, of what can be interpreted as a pH gradient, that was sensitive to vanadate. Further sonication times up to 15 min, did not produce any change in the quenching of the fluorescence. However, this minimal sonication time varied from one experiment to another; besides, it was found that the mixture of liposomes plus the plasma membranes became translucent at a sonication time that varied between 5 and 10 minutes. For this reason, and because of

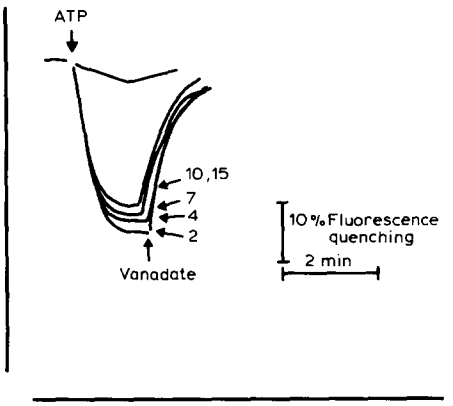

Fig. 1. Effect of the sonication time on the ability of the vesicles prepared to generate a pH gradient with ATP, as monitored with 1.5 μM aminochloromethoxyacridine. The vesicles (400 μg of protein in a final volume of 2.0 ml) were incubated in 20 mM sucrose, 1.5 mM $MgSO_4$, 10 mM Mes-triethanolamine buffer (pH 6.5) in the presence of 100 mM $NaNO_3$. The reaction was started by the addition of 1.5 mM ATP. The mixture of plasma membranes and liposomes was frozen and shawed twice, before sonication during the indicated times. Vanadate concentration was 100 μM. The fluorescence was followed at 400–440 nm.

the difficulty to reproduce perfectly constant conditions, a sonication time of 10 min was chosen thereafter as the standard procedure for the preparation of the vesicles. The quenching of the fluorescence of aminochloromethoxyacridine upon the addition of ATP, on the other hand, indicated that protons, as expected, were pumped into the vesicles, generating a pH gradient, acid inside.

A membrane potential, positive inside, detected by the fluorescence changes of oxonol V was consistently observed; it was only slightly inhibited by oligomycin and completely by vanadate, diethylstilbestrol and triphenyltin. Besides, the quenching could be reversed by the uncoupler 1799, and partially by nigericin. These results agreed with the fluorescence changes of aminochloromethoxyacridine produced upon the addition of ATP, indicating the pumping of H^+ into the vesicles, and the generation of a membrane potential, positive inside (results not shown). Nigericin produced also a partial reversion of the quenching, probably by an uncoupling effect (not shown). All these results could be reproduced if the vesicles were prepared and incubated in either 10 mM KCl or 10 mM potassium acetate.

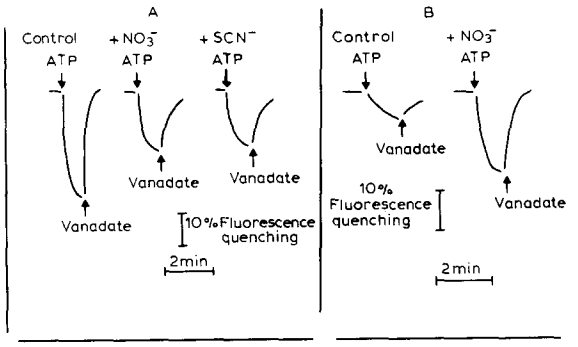

Fig. 2. (A) Effect of NO_3^- and SCN^- on the quenching of fluorescence of oxonol V (580–640 nm) after the addition of ATP to the plasma membrane vesicles of yeast. The vesicles were incubated as described under Methods, in the same medium used for Fig. 1, but with 5 mM $MgSO_4$, and 2 μM oxonol V, and the reaction was started by the addition of 5 mM ATP. Where indicated, 100 mM of either $NaNO_3$ or NaSCN was included in the mixture. Vanadate concentration was 100 μM. (B) Effect of nitrate on the quenching of the fluorescence of aminochloromethoxyacridine by vesicles of plasma membrane from yeast upon the addition of ATP. The experiment was performed as described under Methods, in 20 mM sucrose, 10 mM Mes-triethanolamine (pH 6.5).

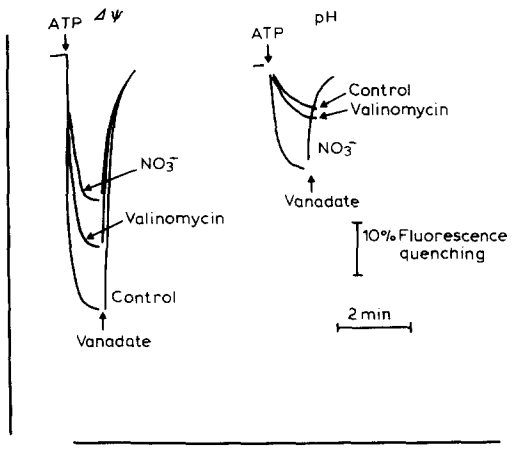

Fig. 3. Effects of valinomycin on the electric potential and pH gradient, as monitored by oxonol V and aminochloromethoxyacridine, respectively, in plasma membrane vesicles from yeast upon the addition of ATP. The experiment was carried out as in Fig. 2, but the vesicles were prepared and incubated in a medium in which 10 mM KCl was used instead of 20 mM sucrose. A tracing with nitrate is included for comparison.

The addition of a permeant anion, like NO_3^-, or SCN^-, produced a decrease of the quenching values of oxonol V, as expected from the ability of these anions to move across membranes with their negative charge (Fig. 2A), neutralizing partially the membrane potential, positive inside. On the other hand, the quenching of aminochloromethoxyacridine that can be interpreted a the result of a pH gradient, was increased by these same anions, which, by relieving the membrane potential, allow the further pumping of protons and higher pH gradients (Fig. 2B). It can be observed that the pH gradient generated was also reversed by the uncoupler 1799. One of the inhibitors of this ATPase that has been reported is DCCD [2,7]. The experiment carried out to determine its action on the generation of an electrochemical potential gradient revealed that, even with large concentrations and with a preincubation of 20 min, no complete inhibition of the pH gradient generated could be achieved (not shown). This agreed partly with a relative insensitivity of the ATPase activity to this ionophore (see below).

Valinomycin, especially if compared to nitrate, produced only a small decrease of the quenching of oxonol V even when the incubation was carried out by preparing and incubating the vesicles in 10 mM KCl (Fig. 3); similar results were obtained with vesicles prepared and incubated in potassium acetate (results not shown). These changes were expected to be at least as large as those observed with nitrate, but they were smaller than those produced by the permeant anion. This behavior of the antibiotic is consistent, however, with only a small stimulating effect (less than 10 percent) on the ATPase activity, observed when the vesicles were prepared in KCl and incubated in a K^+-free medium (not shown).

The experiments to incorporate cytochrome oxidase into the vesicles proved to be successfull; we were fortunate to find that the enzyme preparation used could be inserted in the membrane without the use of detergents, other than the Tween-20 used in the isolation procedure [28]. The experiments to define the characteristics of the insertion of cytochrome oxidase showed a reasonable degree of coupling of respiration. The respiratory ratio with/without 2 μM FCCP varied between 1.8 and 2.5, in comparison to what could be obtained with liposomes prepared from PC without the membranes, in which this ratio could reach a value close to 5.0 [28].

The addition of a substrate (ascorbate/tetramethyl-*p*-phenylenediamine/cytochrome *c*) to the inserted cytochrome oxidase could produce a membrane potential demonstrable by the distribution ratio of tetraphenylphosphonium between the vesicles and the medium (Table III), and the quenching of the fluorescence of dipropylthiacarbocyanine (Fig. 4). Moreover, as expected from the presence of a monovalent cation carrier from the plasma membrane, this potential could be partially reversed by K^+ and Rb^+, but not by Na^+ or NH_4^+. Both the quenching of fluorescence and the uptake of tetraphenylphosphonium were reversed by FCCP and CN^- (Table III, Fig. 4).

The transport of monovalent cations was studied in two different ways; the first one consisted in measuring the efflux of K^+ from vesicles loaded with this cation, by means of a K^+ electrode. Fig. 5 shows that the vesicles were reasonably sealed; after a rapid initial increase of the K^+ concentration in the medium, due probably to the ion already out of the vesicles, a slow efflux was observed. The presence of ATP-Mg in the medium

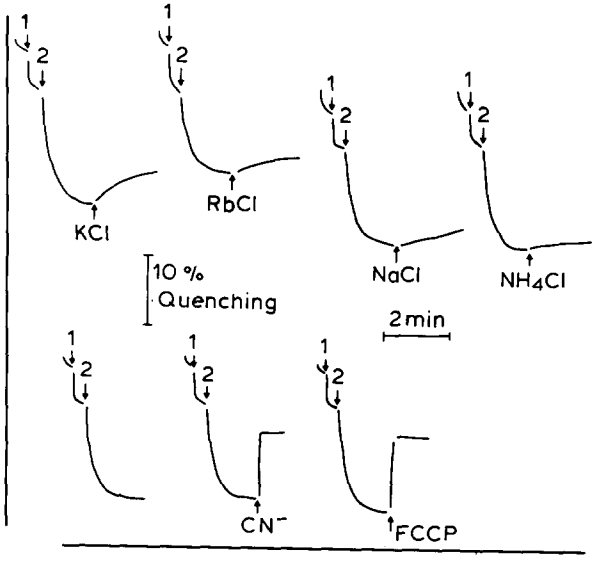

Fig. 4. Detection of a membrane potential in yeast plasma membrane vesicles with cytochrome oxidase incorporated; effects of monovalent cations. After sonication of the vesicles, 375 μg of plasma membrane protein was added per ml, and the mixture was frozen in liquid nitrogen. After thawing at room temperature, the mixture was sonicated for 5 min. Then 25 μl (170 μg) of cytochrome oxidase in 50 mM potassium phosphate buffer (pH 7.0), 0.5% Tween 20 were added. The incubation mixture was: 50 mM phosphoric acid-triethanolamine (pH 7.0), 40 μl (15 μg of plasma membrane protein), in a final volume of 2.0 ml, room temperature. 0.5 μM dipropylthiacarbocyanine was added, and then 200 μg cytochrome *c* at (1), and the reaction was started by the addition of 3 mM ascorbate-triethanolamine (pH 7.0) and 0.3 mM tetramethyl-*p*-phenylenediamine at (2). At the indicated times either 5 mM monovalent cations, 1 μM FCCP, or 200 μM NaCN were added. Fluorescence was followed at 540–580 nM.

TABLE III

UPTAKE OF TETRAPHENYLPHOSPHONIUM BY PLASMA MEMBRANE VESICLES FROM YEAST WITH CYTOCHROME OXIDASE INCORPORATED

The vesicles were prepared and incubated as described for Fig. 4, during 5 min, in the presence of 2 μM ^{3}H-tetraphenylphosphonium and the indicated additions. 500 μl was the final volume and 7.5 μg of vesicle protein were used. After incubation, the amount of radioactive tetraphenylphosphonium remaining in the vesicles was measured as described under Methods.

Condition	nmol·mg^{-1}
Complete system	10.9
No ascorbate	2.1
400 μm NaCN	2.1
1 μM FCCP	2.4
5 mM KCl	4.5
5 mM RbCl	6.9
5 mM NaCl	9.1
5 mM ammonium	11.3

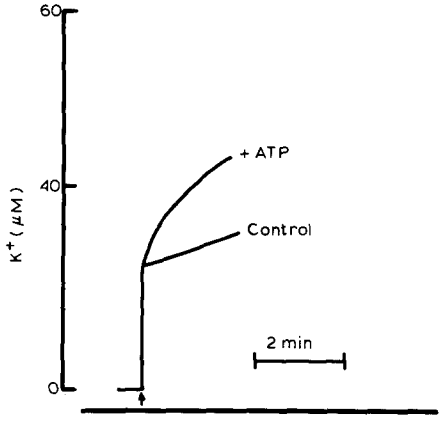

Fig. 5. Effect of ATP on the efflux of K^+ in yeast plasma membrane vesicles. Vesicles were prepared as described for Fig. 1, in 50 mM KCl, and passed through a column of 15 × 0.7 cm of Sephadex G-50. Then, 200 μg of membrane protein were added where indicated, to a final volume of 5.0 ml of 10 mM Mes-triethanolamine (pH 6.5), 100 mM sucrose, containing 1 mM ATP-Mg where indicated. The K^+ concentration changes were followed with a K^+ electrode (Orion).

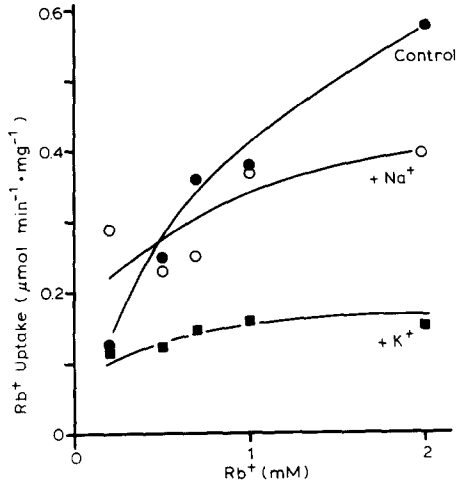

Fig. 6. Uptake of $^{86}Rb^+$ by yeast plasma membrane vesicles with cytochrome oxidase incorporated; Effects of K^+ and Na^+. Vesicles were prepared and incubated as described for Fig. 4, but the indicated concentrations of RbCl were used, and 7.5 μg of vesicle protein (20 μl) were used in a final volume of 500 μl. Incubation lasted for 10 min, at the end of which, the mixture was passed by centrifugation through a Sephadex G-50 column and the radioactivity remaining in the vesicles was counted as described under Methods. When present, the concentrations of sodium and potassium ions were 5 mM and 0.5 mM, respectively.

produced an increased efflux of the cation, probably because of the membrane potential, positive inside, generated by the membrane ATPase.

In another test, the vesicles to which cytochrome oxidase was incorporated, were assayed for their ability to take up $^{86}Rb^+$. The process could be energized by the addition of ascorbate, and inhibited by an uncoupler (not shown). Finally, it was also confirmed that the uptake process was saturable, as shown by the data of Fig. 6. This figure shows also that Rb^+ transport was inhibited by K^+ and to a lesser extent by Na^+, as expected from the properties of the monovalent cation carrier in yeast [34].

Discussion

The preparation used in this work, showed properties similar to those described by Franzusoff and Cirillo [22], except for the fact that, in our hands, it showed activities of phosphatase and pyrophosphatase, that could be removed by an additional centrifugation. The activity seemed to be due to the plasma membrane ATPase, since it was inhibited by vanadate and diethylstilbestrol contrary to the vacuolar enzyme, and not by oligomycin, which distinguished it from the mitochondrial activity [7,36,37]. The activity of the preparation was similar to that of other reported preparations [7,22,23].

Since the preliminary experiments, it was found that the vesicles could generate and maintain a diffusion potential, when loaded with KCl and in the presence of valinomycin (not shown). This fact, added to the sensitivity of the ATPase of the vesicles to FCCP, nigericin and lysophosphatidylcholine, suggested already that the vesicles were more or less sealed, and that the enzyme might be working as a proton pump in this preparation, to generate a membrane potential and a proton gradient.

Only a small activation of the ATPase activity was observed when the uncouplers and other ionophores were added; this could be due to a poor sealing of the membranes; however, this is probably not so, for several reasons: (a) the vesicles, when loaded with K^+, could maintain for several minutes a diffusion potential; (b) the efflux of K^+ shown in Fig. 5 was slow; (c) a potential gradient could be generated both by ATPase and cytochrome oxidase, and (d) ion transport could be demonstrated (Figs. 5 and 6). Also small stimulations by ionophores and uncouplers were reported for this ATPase when reconstituted into liposomes [11]. It is possible that the low stimulation produced by uncouplers was due to an inadequate insertion of the ATPase in the vesicles, or also to ion transport systems present in the preparation (of which at least monovalent cation transport could be demonstrated).

Experiments, performed to define the characteristics of the electrochemical potential difference generated by ATP between the inside and the outside of the vesicles, confirm that the electrochemical potential difference consists of an electric component, positive inside, as well as a pH gradient, acid inside the vesicles. Besides, the addition of permeant anions was found to decrease the positive potential generated by ATP, and this coincided with an increase of the pH gradient. These results indicated that the ATPase

was at least partially coupled and capable of generating a membrane potential.

The membrane preparation appears to have some advantages over that reported by Fuhrmann et al. [21], as well as that of Peters and Borst-Pauwels [38]. Those preparations were tried and found to have a low ATPase activity (not shown), and it was not possible to generate a membrane potential in them, most probably because they were leaky. Another vesicle preparation from the plasma membrane of *Saccharomyces cerevisiae* has been reported by Ahlers [23], and it seems also adequate to be used in solute transport studies.

The possibility to incorporate cytochrome oxidase into the vesicles made more interesting this membrane preparation, especially because the procedure was found to be extremely simple; in fact, it was only necessary to mix the enzyme with the vesicles to get a reasonable degree of incorporation of the functional enzyme, that was also capable of generating a membrane potential, negative inside, which could be verified both by the fluorescence quenching of dipropylthiacarbocyanine and the distribution of tetraphenylphosphonium. This is a very convenient way to develop a membrane potential that has the same polarity observed in the whole cell [7,20], and might be used to drive ions inside, as postulated for yeast [1,7,16].

The study of ion movements seems to confirm that the carrier for monovalent alkali cations is present in these vesicles. This was indicated by the following facts: (a) the increased efflux of K^+ produced in the vesicles by the addition of ATP; (b) the uptake of Rb^+, apparently driven into the vesicles by the membrane potential generated by cytochrome oxidase, (c) the saturability of this system by Rb^+; (d) the possibility to inhibit this transport more or less selectively by K^+, and to a much lower degree by Na^+, and (e) the fact that K^+ and Rb^+, but not Na^+, were able to produce a decrease of the membrane potential, as seen both by the quenching of fluorescence of the cyanine, and by the decrease of the uptake of tetraphenylphosphonium by the vesicles with cytochrome oxidase incorporated. However, the value of kinetic constants is difficult to determine from our experiments. Several factors may influence the results, such as the presence of other carriers, and the orientation of the yeast membrane fragments in the vesicles, which most probably is produced at random during the fusion process.

The preparation with the incorporated cytochrome oxidase can also be interesting, because it shows that monovalent cations (Rb^+) can be taken up in the absence of ATP, just by generating a membrane potential. As in intact cells, transport of monovalent cations appeared to be driven by a membrane potential; the transport was selective and seemed more or less saturable. Besides, as shown in the vesicles with cytochrome oxidase incorporated, it might not necessarily be coupled to the hydrolysis of ATP in an exchange for H^+ [39].

In summary, the results obtained in this work are an indication that this preparation may be used as an adequate model to start the study of ion transport in vesicles of plasma membrane of yeast, that seem to preserve the components of at least some of the solute transport systems present in the cell.

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