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SUGAR TRANSPORT AND POTASSIUM PERMEABILITY IN YEAST PLASMA MEMBRANE VESICLES

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SUMMARY

Plasma membrane vesicles were isolated from homogenised yeast cells by filtration, differential centrifugation and aggregation of the mitochondrial vesicles at pH 4. As judged by biochemical, cell electrophoretic and electron microscopic criteria a pure plasma membrane vesicle preparation was obtained.

The surface charge density of the plasma membrane vesicles is similar to that of intact yeast cells with an isoelectric point below pH 3. The mitochondrial vesicles have a higher negative surface charge density in the alkaline pH range. Their isoelectric point is near pH 4.5, where aggregation is maximal.

The yield of vesicles sealed to K^+ was maximal at pH 4 and accounted for about one third of the total vesicle volume.

The plasma membrane vesicles demonstrate osmotic behaviour, they shrink in NaCl solutions when loosing K^+.

As in intact yeast cells the entry and exit of sugars like glucose or galactose in plasma membrane vesicles is inhibited by UO_2^{2+}.

Counter transport in plasma membrane vesicles with glucose and mannose and iso-counter transport with glucose suggests that a mobile carrier for sugar transport exists in the plasma membrane.

After galactose pathway induction in the yeast cells and subsequent preparation of plasma membrane vesicles the uptake of galactose into the vesicles increased by almost 100 % over the control value without galactose induction. This increase is explained by the formation of a specific galactose carrier in the plasma membrane.

INTRODUCTION

Since 1960 plasma membrane vesicles have been used in the study of transport mechanisms for small molecules in bacteria [1]. The most important advantage of these wall-less vesicles is that transport can be examined without interference from cellular metabolism. The possibility of genetic manipulations is a further advantage for examination of the biochemistry of transport.

In a previous publication [2] we described a mechanical preparation of yeast

plasma membrane vesicles and their identification. These vesicles are oriented right side out and in contrast to bacterial vesicles have no respiration functions.

For the present investigation of sugar transport and K^+ permeability the previously described vesicle preparation was modified in order to increase the quantity and the quality of sealed plasma membrane vesicles. The presence and the induction of sugar carriers in the plasma membranes will be discussed.

MATERIALS AND METHODS

Vesicle preparation. As previously described [2] we used starved baker's yeast (*Saccharomyces cerevisiae*, Hefefabriken Hindelbank AG, Bern) for our experiments. For induction of galactose pathway enzymes and galactose transport 0.6 wet vol. % yeast cells were incubated with 300 mg % glucose, 300 mg % galactose, 2 % peptone and 1 % yeast extract at 30 °C [3] for the given time periods. After induction the cells were washed about 10 times with distilled water until no bacterial contamination was visible by microscopic examination.

The previously described method of vesicle preparation [2] was modified. Instead of grinding the yeast cells by hand, a Cell Homogeniser MSK (Braun Mel-

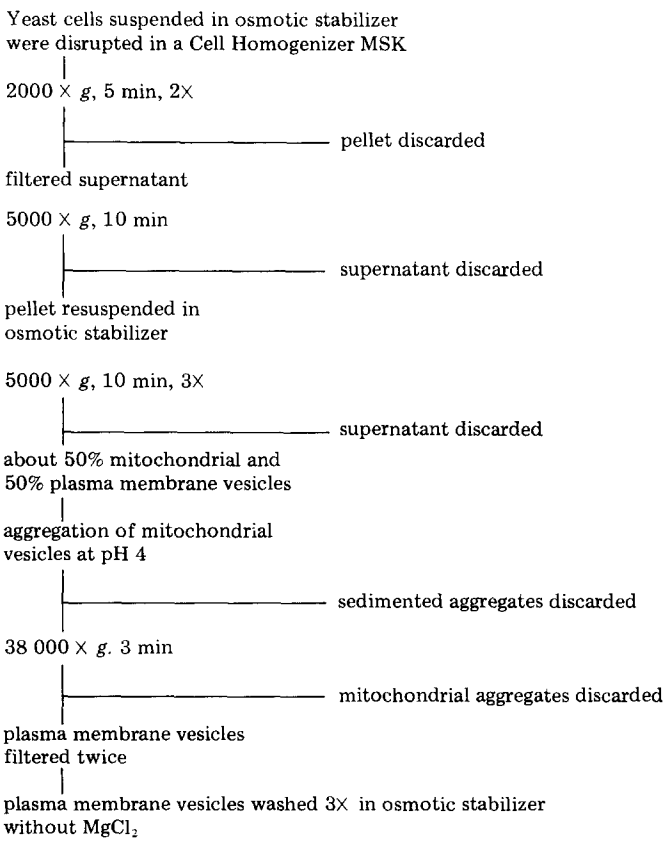

Fig. 1. Scheme of vesicle preparation.

sungen, G.F.R.) was used. The scheme of preparation is shown in Fig. 1. 20 g washed cells were suspended in 20 ml ice-cold osmotic stabilizer solution (400 mM KCl, 1 mM $MgCl_2$ and 20 mM triethanolamine, adjusted to pH 4) and homogenized with 50 g glass beads (0.25–0.30 mm diameter) for 45 s at 4000 rev./min. This changed the pH of the solution to about pH 6. The homogenate was centrifuged twice in a Sorvall RC2-B centrifuge for 5 min at $2000 \times g$ and the pellet, consisting of broken and unbroken cells, discarded. The $2000 \times g$ supernatant was filtered (glass fiber filter Sartorius, 13400, Membranfilter GmbH, G.F.R.) to remove remaining cells and spun at $5000 \times g$. Thereafter the supernatant was discarded and the vesicle pellet resuspended in osmotic stabilizer solution and washed three times at $5000 \times g$ for 10 min. Instead of the previously used sucrose density gradient, we separated the remaining plasma membrane and mitochondrial vesicles by aggregation of the mitochondria. Therefore the pH of the vesicle suspension (about pH 5.5) was brought to pH 4 with an osmotic stabilizer solution adjusted to pH 1.2 with 1 M HCl. At this pH only the mitochondrial vesicles aggregate and start to sediment. After 20 min the white plasma membrane vesicles contaminated by small mitochondrial aggregates can be pipetted from the sedimented brownish mitochondrial aggregates. A further purification of the plasma membrane vesicles is obtained after 3 min centrifugation at $38\,000 \times g$ by removing the upper layer of white plasma membrane vesicles and leaving aggregated mitochondria at the bottom of the tube. Finally the plasma membrane vesicles were filtered twice through the 13400 Sartorius Membranfilter and washed three times at $5000 \times g$ in osmotic stabilizer solution without $MgCl_2$.

Cell electrophoresis. Cell electrophoretic mobilities were measured at 25 °C using a rectangular cuvette and apparatus described by Fuhrmann et al. [4]. The composition of the buffer solution was 2.85 mM sodium acetate, 2.85 mM sodium veronal, 70 mM KCl and 600 mM manitol and the pH was adjusted with HCl. From electrophoretic mobility the zeta potential was calculated by using the Helmholtz-Smoluchowski equation [5] and the surface charge density was estimated by means of the Gouy-Chapman equation [6]. The capability of the system was demonstrated by determining the velocity parabola (mobility versus depth of the cuvette). From 40 measurements on human erythrocytes in 145 mM NaCl solution at pH 7.2 and 25 °C the surface charge density was, 3715 e.s.u./cm^2, which is in agreement with the values reported in the literature [7].

Electron microscopy. For freeze-fracturing the vesicles were pelleted in osmotic stabilizer solution pH 4 containing 30% glycerol and frozen in liquid freon 22. Freeze-fracturing was performed in a Balzers BA 360 apparatus after using a slightly modified procedure of Moor [8]. Fracturing was done at −110 °C immediately followed by shadowing.

Permeability studies. Vesicle volume was determined by a cytocrit ($12\,000 \times g$, 15 min). K^+ efflux was measured in 400 mM NaCl with and without divalent cations and 20 mM triethanolamine adjusted to the given pH and O °C (ice bath). The vesicle sediment was rinsed twice with the NaCl solution to remove outside K^+ and the experiment was started after addition of the NaCl solution and suspension of the vesicles. Samples were taken by centrifugation ($15\,000 \times g$, 3 min) and the sedimented vesicles lysed in distilled water by freezing. After centrifugation the supernatant was analysed for K^+ with a flame photometer Model 143 (IL Instrumentation Laboratory Inc. Lexington/Massachusetts, USA). K^+ at the beginning of the experiment was

calculated from 1 part of vesicle solution diluted 10 times with distilled water after freezing.

Sugar uptake and exit in plasma membrane vesicles was measured by using ^{14}C-labelled sugars and millipore filter technique [9]. 0.1 ml samples were diluted in 5 ml ice-cold stopper solution (425 mM KCl, 0.01 mM uranyl nitrate and 20 mM triethanolamine, pH4), filtered and rinsed with 5 ml stopper solution on the filter (0.6 μ Sartorius Filter, G.F.R., 1.5–2.5 μl vesicles per filter, suction pressure 20 cm Hg).

Analytical methods. O_2 uptake was measured as described previously [2].

CO_2 production was measured under anaerobic conditions (N_2) by the Warburg technique.

Proton change was estimated by means of a pH-stat (Radiometer Copenhagen) of the vesicle suspension (400 mM NaCl solution and 0.5 wet vol. % vesicles).

Chemicals. D-[^{14}C]Glucose and D-[^{14}C]galactose (Amersham, Buckinghamshire, U.K.). All other chemicals were A.R. reagents.

RESULTS

Characterisation of plasma membrane vesicles

After freeze-fracturing yeast plasma membranes exhibit characteristic invaginations and hexagonal arrangements of particles [2, 10]. Fig. 2 shows a freeze-fracture replica of plasma membrane vesicles isolated as described above. In contrast to the previously described plasma membrane vesicles [2] the invaginations of these vesicles are reduced (i1 in Fig. 2) or even reversed (i2 in Fig. 2). This difference is due to the acidic medium used (pH 4) to separate the mitochondria from the plasma membrane vesicles by aggregation of the mitochondria. The freeze-fractured mitochondria pellet of Fig. 3 shows an almost pure preparation of mitochondria.

In respect to surface charge plasma membrane vesicles differ considerably from mitochondrial vesicles, but not from intact yeast cells (Fig. 4 and 5). The negative surface charge density of mitochondrial vesicles increases more steeply with pH and is significantly higher in the alkaline pH than that of plasma membrane vesicles or intact yeast cells. The isoelectric point of mitochondrial vesicles is around pH 4.5 whereas the isoelectric point of plasma membrane vesicles and intact yeast cells can be assumed below pH 3. Between plasma membrane vesicles and intact yeast cells a small difference exists of about 500 e.s.u./cm^2.

When measuring aggregation of plasma membrane and mitochondrial vesicles in between pH 3 and pH 7 (electrophoresis buffer) by % change of transmission at 700 nm (Fig. 6) only the mitochondrial vesicles show aggregation which is also clearly visible by eye. The maximal aggregation of the mitochondrial vesicles lies around pH 4.5, which is their isoelectric point.

A further destinction between plasma membrane vesicles and mitochondrial vesicles is that respiration is absent in the first and present in the second. With 2 mM NADH, respiration in the mitochondria was about 200 natom 0/mg protein per min as measured previously [2].

The plasma membrane vesicles did not show any CO_2 or proton production with glucose or galactose as substrate at 28 °C.

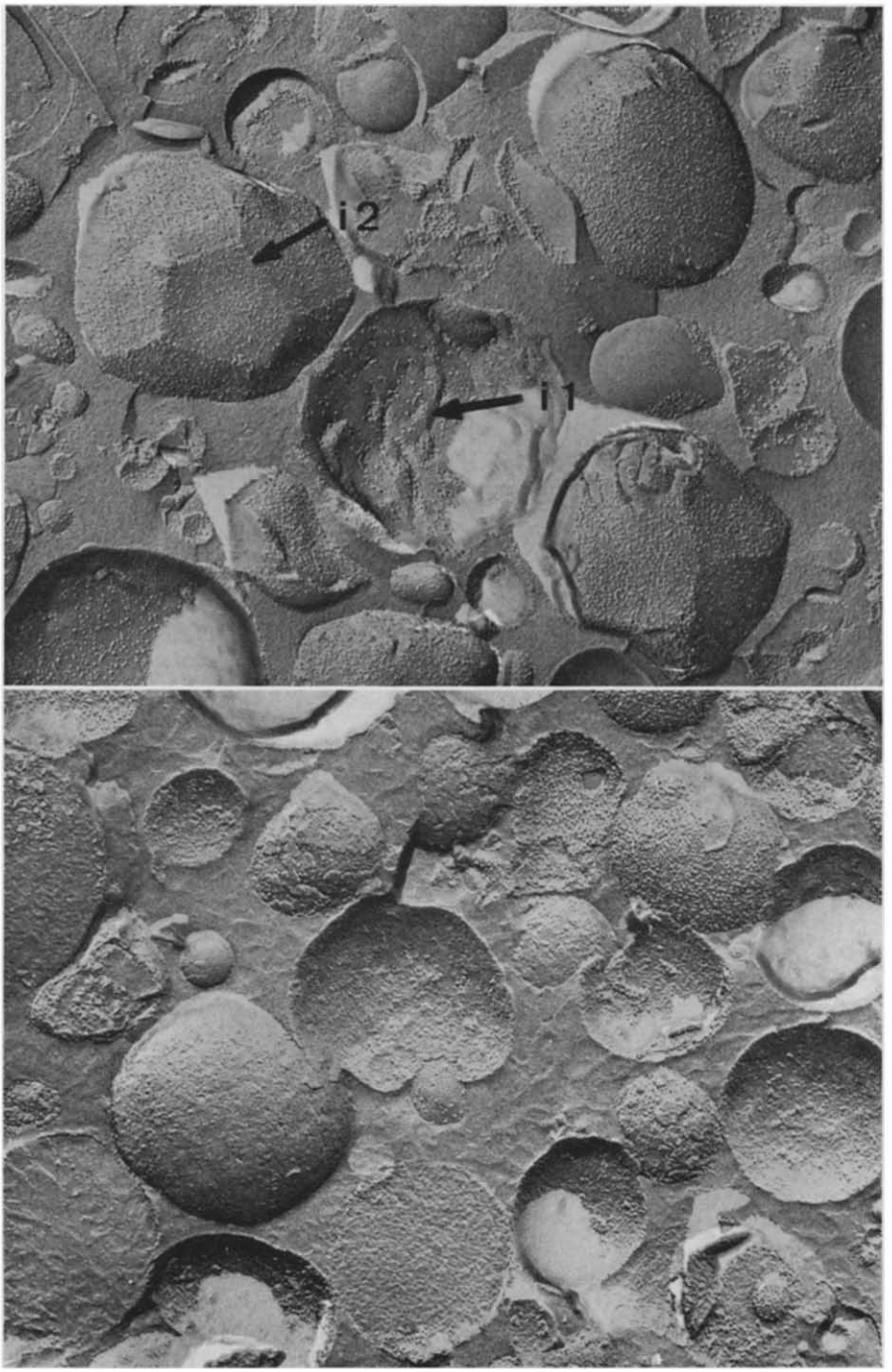

Fig. 2. Freeze-fracture replica of plasma membrane vesicles. i_1 reduced invaginations, i_2 reversed invaginations. × 40 000.

Fig. 3. Freeze-fracture replica of mitochondrial vesicles. × 40 000.

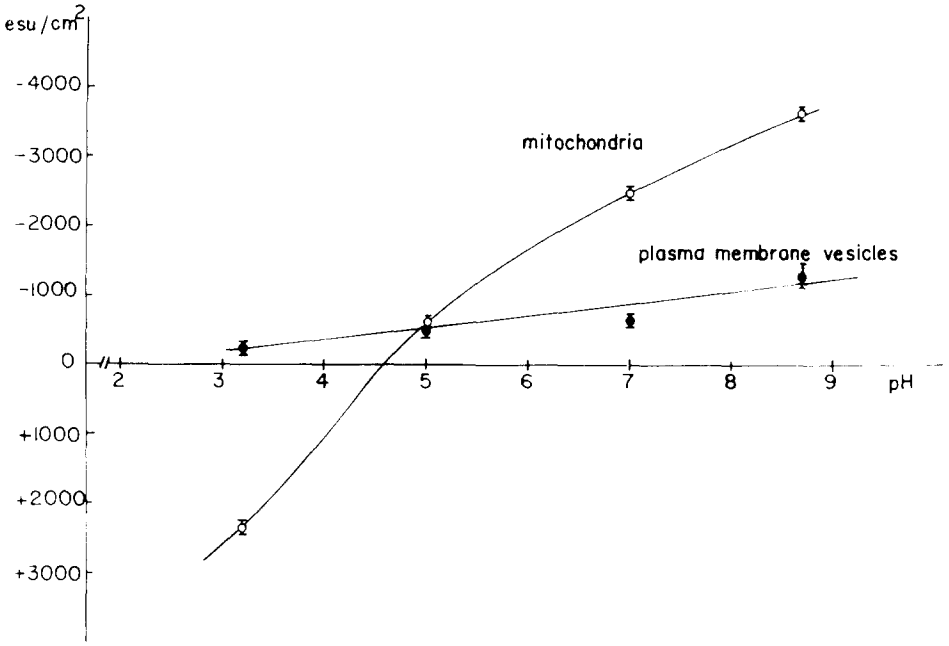

Fig. 4. Surface charge density of plasma membrane vesicles and mitochondria in relation to pH. 20 measurements, results expressed as mean ± S.E.

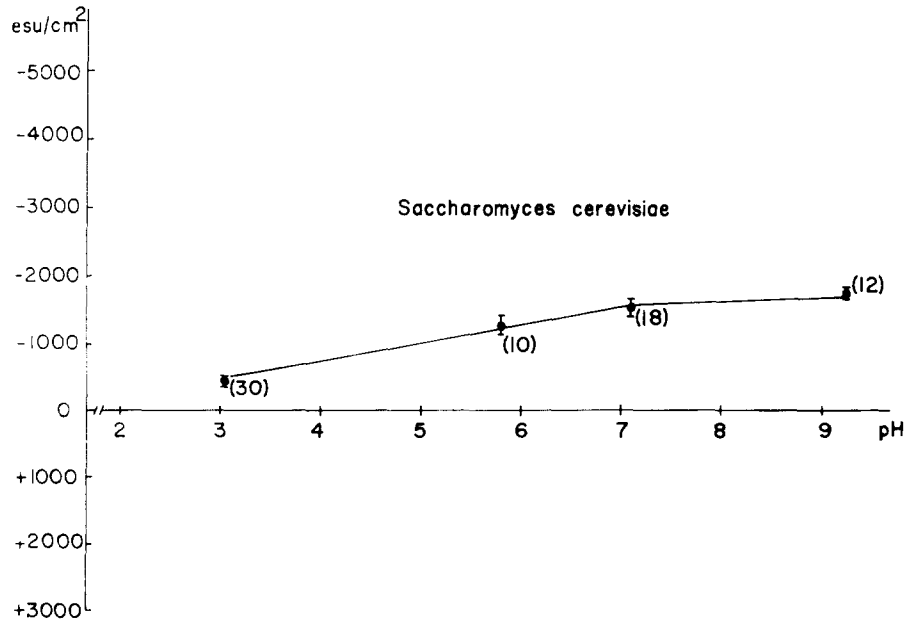

Fig. 5. Surface charge density of yeast cells. Number of measurements is given in brackets and results are expressed as mean ± S.E.

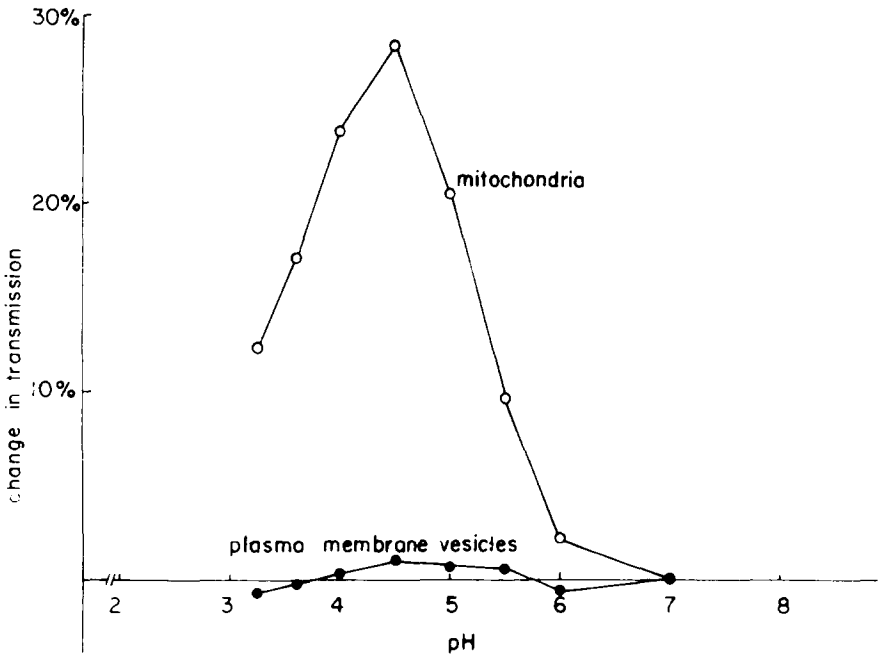

Fig. 6. % change in transmission at 700 nm of plasma membrane vesicles and mitochondrial vesicles suspended in cell electrophoresis buffer.

Permeability studies

K^+ efflux in plasma membrane vesicles was measured at approximately zero K^+ outside and O °C (ice bath). Fig. 7 shows that there is an initial rapid loss of K^+ followed by a low efflux component. The rapid loss is thought to be related to leaky vesicles, whereas the slow component is attributed to sealed vesicles. At pH 4 the yield of vesicles sealed to K^+ is highest, about one third of the total vesicle volume. Any alteration in the pH of the medium significantly decreases the yield of sealed vesicles.

In a further set of experiments the influence of outside added divalent cations like Mg^{2+}, Ca^{2+} or UO_2^{2+} on K^+ efflux in plasma membrane vesicles was tested at pH 4 under similar conditions as described above. Mg^{2+} and Ca^{2+} were applied in a concentration range from 0.2–20 mM and UO_2^{2+} in 0.1 mM. The added divalent cations did not change the yield of sealed vesicles.

The following experiment shows the osmotic behaviour of the plasma membrane vesicles (Fig. 8). The plasma membrane vesicles were incubated for 10 min at room temperature (22 °C) in solutions of the following composition: 400 mM KCl/0 NaCl: 200 mM KCl/200 mM NaCl; 100 mM KCl/300 mM NaCl; 0 KCl/400 mM NaCl. All solutions contained 20 mM triethanolamine adjusted to pH 4 or pH 7. After this time K^+ had equilibrated and the volume of the vesicles was estimated by measuring the cytocrit. The plasma membrane vesicles shrink in sodium-containing media. The highest shrinkage is obtained at pH 4. This result is in accordance with osmotic behaviour of the plasma membrane vesicles and with the interpretation that the

amount of sealed vesicles is maximal at pH 4. During K^+ loss no proton uptake could be detected on a pH stat.

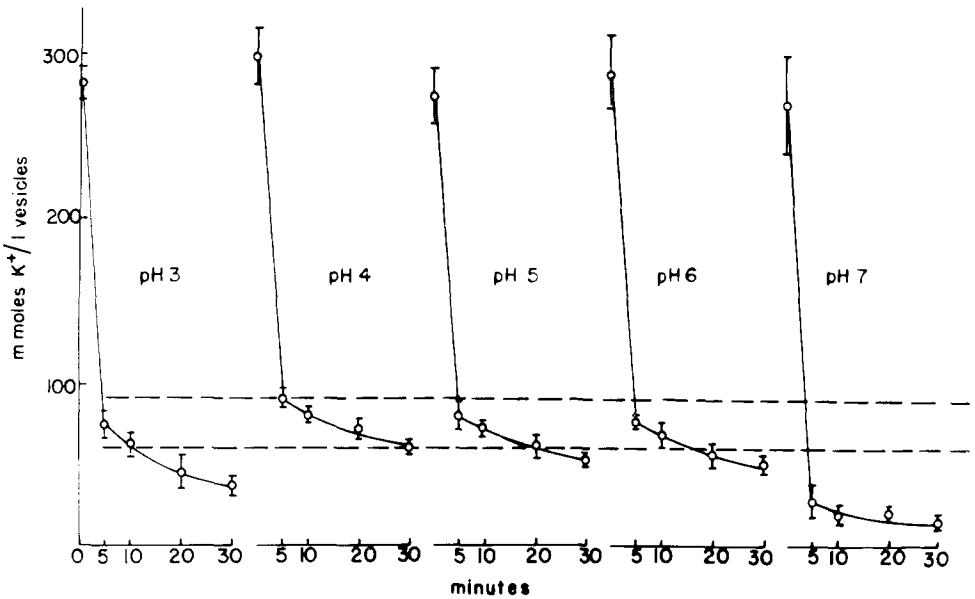

Fig. 7. Dependence of K^+ efflux in plasma membrane vesicles from time and pH. The dotted lines include the data between 5 and 30 min at pH 4. Ice bath temperature and outside K^+ approximately zero. Mean of 6 experiments ± S.E.

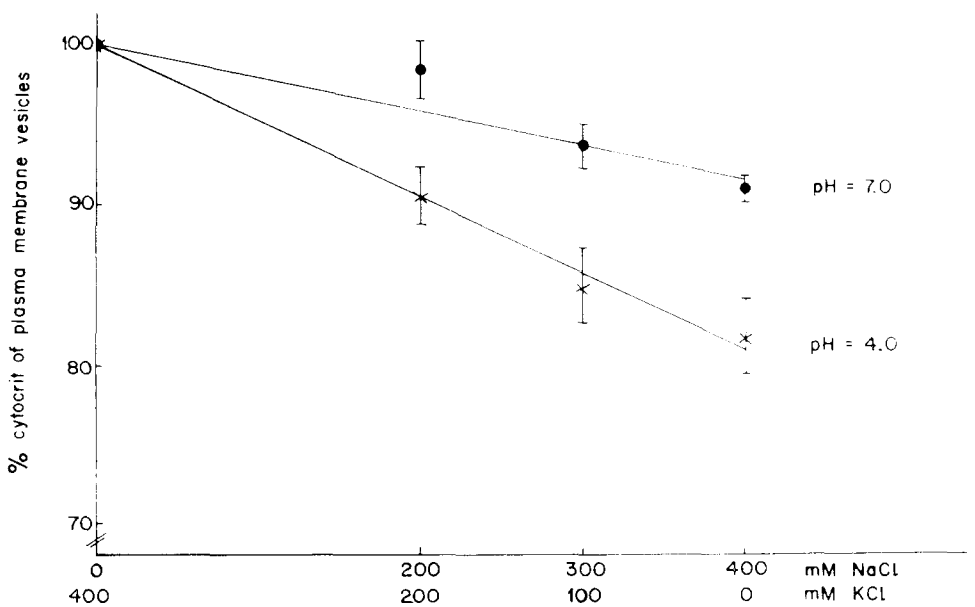

Fig. 8. Cytocrit of plasma membrane vesicles at pH 4 and pH 7 after K^+ exit (10 min at room temperature) in Na^+/K^+ solutions as given on the abscissa. Mean of 6 experiments ± S.E.

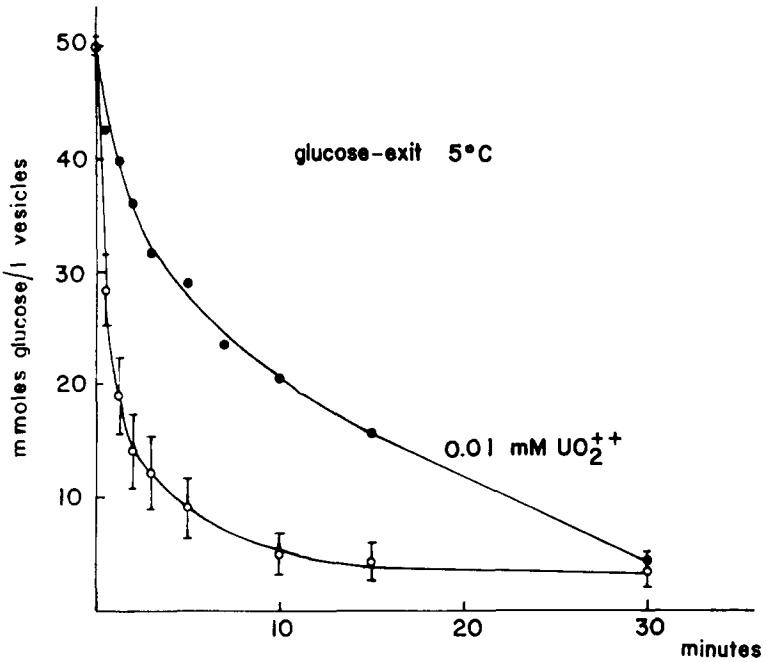

Fig. 9. Exit of glucose in plasma membrane vesicles at approximately zero outside glucose concentration, pH 4 and 5 °C. (○) Mean of 4 experiments ±S.E. without UO_2^{2+} and (●) one experiment with 0.01 mM UO_2^{2+}

Fig. 9 shows that glucose exit at 5 °C from glucose-preloaded plasma membrane vesicles at approximately zero glucose outside in the presence and absence of UO_2^{2+}. The exit of glucose from the plasma membrane vesicles is significantly inhibited by UO_2^{2+}. Similarly the entry of glucose [11] as well as the entry and exit of galactose is inhibited by UO_2^{2+}. We therefore used UO_2^{2+} in the stopper solution to reduce sugar permeation in plasma membrane vesicles during filtration.

In Fig. 10 the phenomenon of counter transport [12] between the sugar pair glucose (at the beginning 10 mM inside and outside the vesicles) and mannose (at the beginning 50 mmol per l vesicles and outside zero) is demonstrated. Glucose is driven out of equilibrium by mannose and the labelled glucose increases in the vesicles and equilibrates again with time as shown by cpm/min.

The phenomenon of counter transport in plasma membrane vesicles is also obvious for isocounter transport of glucose (Fig. 11). The labelled glucose in equilibrium (inside and outside 0.167 mM) is driven out of equilibrium by 50 mmol unlabelled glucose (at the beginning only inside the vesicles). This is shown at 0, 5 and 15 °C. At 0 °C labelled glucose returns to equilibrium after approximately 120 min (not shown).

From CO_2 production with galactose as substrate it is shown that our yeast strain does not contain the enzymes for the galactose pathway (Fig. 12). After induction of the galactose pathway enzymes in the yeast cells CO_2 production with galactose increases at about the time when glucose is used up from the induction medium.

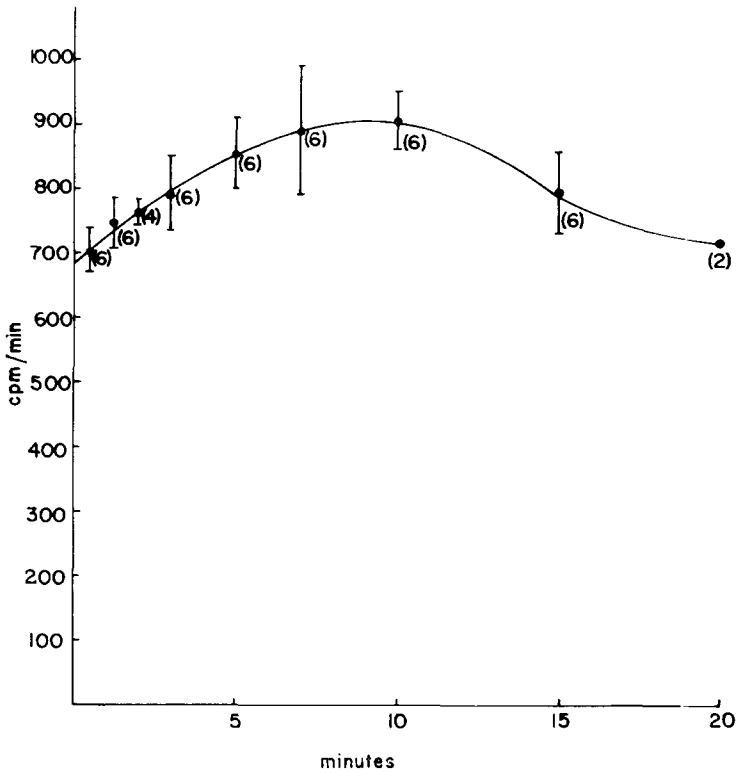

Fig. 10. Counter transport in plasma membrane vesicles with the sugar pair glucose-mannose at pH 4 and 0 °C. The number of experiments is given in brackets, results as mean ±S.E.

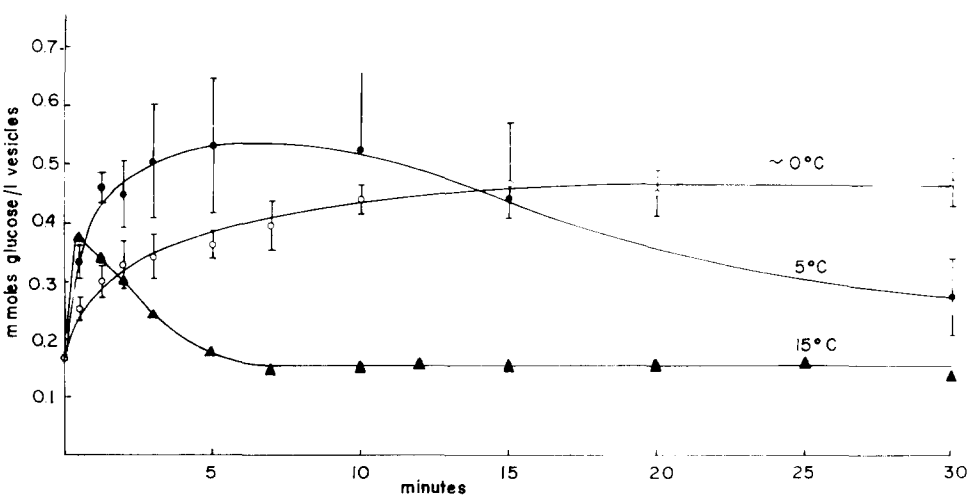

Fig. 11. Iso-counter transport of glucose in plasma membrane vesicles at three different temperatures (0,5 and 15 °C) and pH 4. At 0 °C mean of 6 experiments ±S.E., at 5 °C mean of 4 experiments ±S.E. and at 15 °C mean of two experiments.

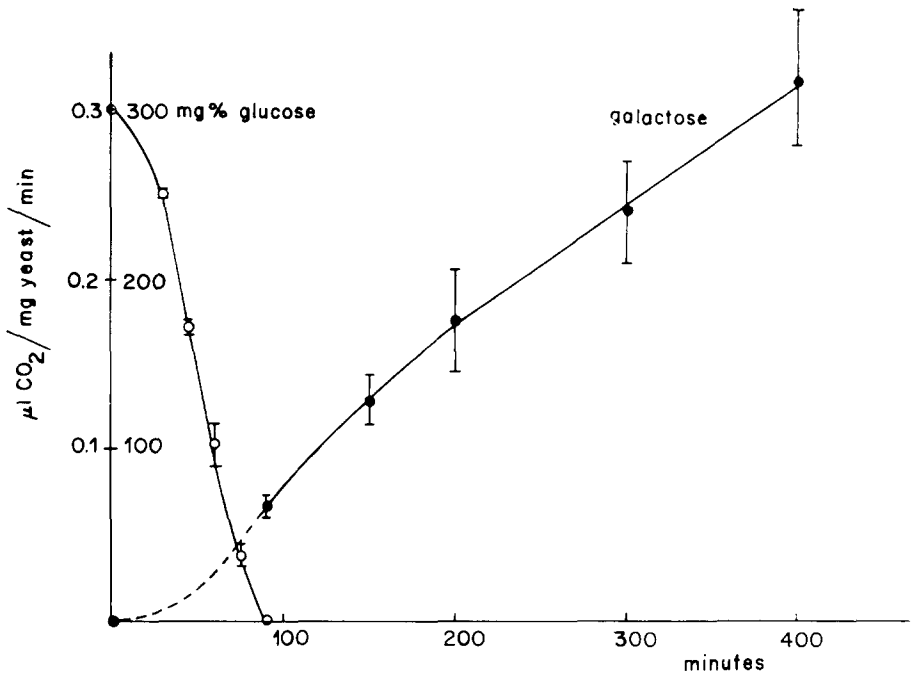

Fig. 12. (●) CO_2 production of yeast cells from galactose after different time periods of galactose pathway enzyme induction. After induction the yeast cells were washed and tested in the Warburg vessel (10 mg yeast, 5 mM galactose, pH 5 and 28 C) under anaerobic conditions (N_2) for CO_2 production. Mean of 6 experiments ±S.E. (○) Disappearance of glucose from the induction medium. Mean of 6 experiments ±S.E.

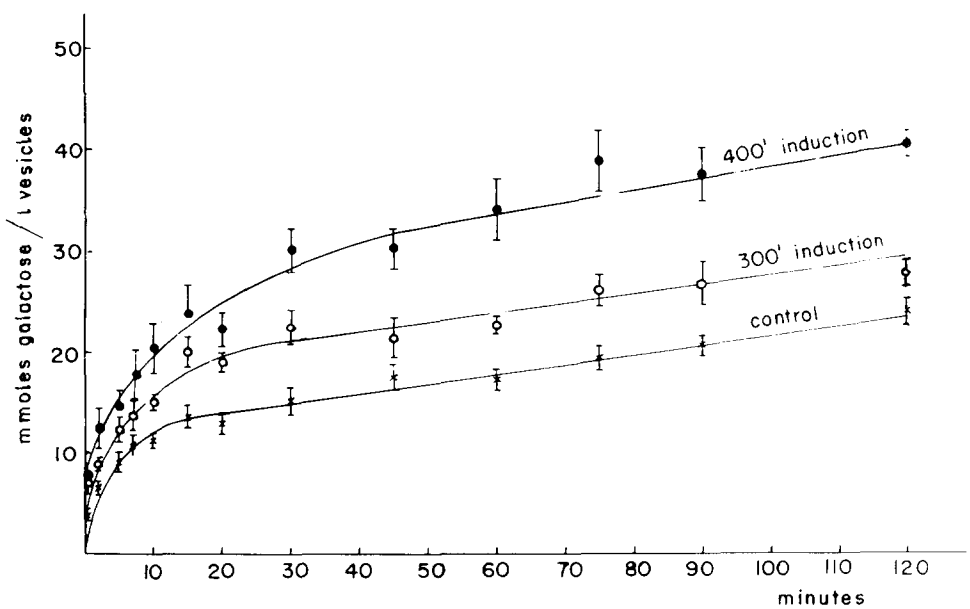

Fig. 13. Galactose uptake in plasma membrane vesicles at 0 C and pH 4. Plasma membrane vesicles have been prepared after the given time periods for induction of galactose pathway enzymes in the intact yeast cells. Mean of 6 experiments ±S.E.

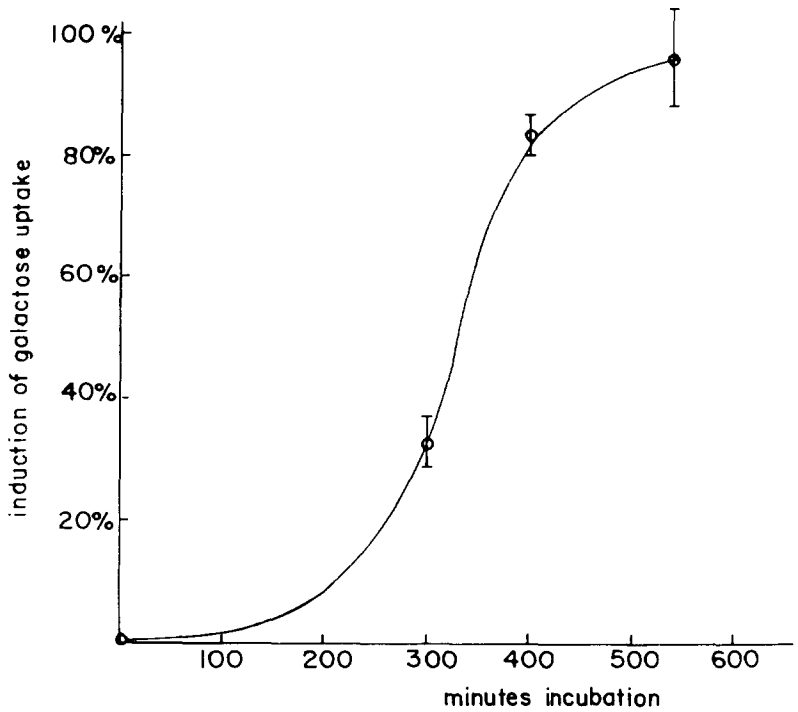

Fig. 14. % change of galactose uptake over the control in relation to the induction period. Mean and S.E., were calculated from all single points in Fig. 13. The experiment at 540 min is a double experiment not shown in Fig. 13. In this experiment we added a further 300 mg % galactose at 250 and 400 min.

When preparing plasma membrane vesicles from induced yeast cells an increase in galactose uptake in the vesicles is clearly demonstrable (Fig. 13). After 300 min of induction the increase over control was 37.6 ± 4.6 %, after 400 min induction 83.2 ± 3.8 % and after 540 min 95.6 ± 8.9 % (Fig. 14, the last experiment is not shown in Fig. 13). Incubation of yeast cells in the induction medium but without galactose has no effect on the rate of galactose uptake into plasma membrane vesicles prepared from these cells.

DISCUSSION

In a recent paper [2] we described preparation and identification of yeast plasma membrane vesicles by mechanical disruption of yeast cells and separation of the plasma membrane vesicles from mitochondrial vesicles on a sucrose density gradient. However, the intactness of the plasma membrane vesicles in respect to transport functions such as K^+ permeability and glucose transport, was difficult to establish because of the low quantity of vesicles and the low yield of sealed vesicles. By changing from grinding of the yeast cells by hand to homogenisation with a Cell Homogeniser MSK the vesicle yield was increased by a factor of about 20. The quality of the vesicles was improved by avoiding the osmotic stress of a sucrose density gra-

dient and by taking advantage of differences in surface charge of the two kinds of isolated vesicles.

From cell electrophoresis it is obvious that the plasma membrane vesicles differ in their surface charge density and isoelectric point from mitochondrial vesicles. The mitochondrial vesicles aggregate around their isoelectric point at pH 4.5. This aggregation is connected with a high sedimentation rate which could be used in separation from the plasma membrane vesicles. By this separation technique one obtains pure plasma membrane vesicles and pure mitochondrial vesicles which are shown in Fig. 2 and 3. The surface charge of intact yeast cells is similar to that of plasma membrane vesicles. It could be speculated that in intact yeast and plasma membrane vesicles strong negative groups, such as phosphoryl groups, as suggested by Rothstein from binding studies [13], are responsible for the low isoelectric point below pH 3.

The vesicles have been characterized not only by surface charge density and morphology but also by biochemical tests. Respiration was completely absent from plasma membrane vesicles whereas the mitochondrial vesicles demonstrated respiration as previously shown [2]. Metabolic functions as CO_2 or proton production from glucose or galactose could not be detected in isolated plasma membrane vesicles. Thus, interference from metabolism in transport studies seems to be unlikely.

From our transport studies in plasma membrane vesicles it can be concluded that the membrane of the isolated vesicles acts as a permeability barrier towards monovalent cations and sugars. At least part of the vesicles are sealed for K^+, showing a slow exit rate for this cation. The shrinkage of plasma membrane vesicles in Na^+ media indicates that the Na^+ permeability is smaller than the K^+ permeability. Since the relationship between the efflux and influx pathways for cations in intact yeast cells have not yet been clearly established the vesicles may be a valuable tool for the investigation of cation movements across yeast cell membranes. This topic is under investigation in our laboratory. The influence of pH on the amount of vesicles sealed to K^+ points to an important effect of charged groups in the membrane for the control of cation permeability. Similar influences of pH have been reported for resealing in red cell ghosts [14].

The plasma membrane vesicles are also only partly sealed for sugars. This can be inferred from the counts of labelled sugars at the end of sugar entry into the vesicles. From the data it is apparent that at pH 4 the fraction of vesicles sealed for glucose and galactose is similar in magnitude to the fraction which sealed for K^+. The entry and the exit of the sugars can be inhibited by UO_2^{2+}. In this respect vesicles behave like intact yeast cells in which the sugar entry [15] and exit [16] is also inhibited by UO_2^{2+}.

The phenomenon of counter transport of sugars in the vesicles points to the existence of a mobile carrier in the plasma membrane [12]. The unequal distribution of one sugar species builds up a carrier gradient in the plasma membrane which drives the second sugar out of equilibrium. This process is strongly temperature dependent and is also inhibited by UO_2^{2+}.

The plasma membrane vesicles are suitable for the investigation of mutation effects on transport. Our yeast cells do not possess the enzymes for the galactose pathway since there is no CO_2 production on addition of galactose. After induction of the galactose pathway in intact yeast cells [3] and subsequent measurement of CO_2 production on addition of galactose the increase of CO_2 production is taken as a positive correlation

for induction. Plasma membrane vesicles derived from the yeast cells before induction or pretreated in the induction medium without galactose show a low uptake of galactose. This result is in agreement with observations on intact bakers yeast deficient in the structural gene for galactose transport [17]. It has been shown that phosphorylation is not a requisite step in transport of galactose in intact bakers yeast even after induction and that the transport occurred via facilitated diffusion [18]. The nature of this system, leading to equilibration, distinguishes it from such inducible transport systems as in bacteria which are uphill and energy dependent [19]. Preparation of vesicles after induction of the galactose pathway enzymes in intact yeast resulted in a significant increase of galactose uptake in the vesicles. It is interesting to note that increase of galactose uptake over the control is S-shaped in relation to the time of induction, whereas increase of CO_2 production in the intact yeast cells is nearly linear with time after a latence period at the beginning. The former result points to a saturation of the plasma membrane with a galactose carrier which might be a protein. Experiments on changes of the protein patterns before and after galactose induction and their relationship to the transport are under investigation.

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