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Workshop on Yeast Transport and Energetics

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264 Workshop on Yeast Transport and Energetics

Fundación Juan March

Serie Universitaria

264

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I N D E X

PROGRAMME

Monday 16: *CATION TRANSPORT AND CHANNELS*
Chairman: A.A. Eddy

A. Peña - *A possible gene coding for K^+ transport in Kluyveromyces lactis.*

R. Lagunas - *Turnover of the K^+ transport system in Saccharomyces cerevisiae.*

H.P. Bode - *Accumulation of strontium by vacuolar protein targeting mutants of Saccharomyces cerevisiae.*

A. Bertl - *Ion channels in Saccharomyces cerevisiae.*

V. Vacata - *Multiconductance ionic channels in the plasma membrane of the yeast Schizosaccharomyces pombe.*

REGULATION OF CATION TRANSPORTS
Chairman: Y. Eilam

J. Ramos - *Glucose activation of the potasium uptake system and plasma membrane H^+ -ATPase of yeast. Relationship with the c AMP pathway.*

R.L. Brandao - *Mechanism of glucose-induced activation of plasma membrane H^+ -ATPase in cells of the yeast Saccharomyces cerevisiae.*

J.I. Castrillo - *The chemostat. A valuable tool for the in vivo determination of the specific rate of proton production (qH^+) from yeast cells.*

General discussion on cation transports. Chairman: A. Peña.

ATPases
Chairman: D. Kuschmitz

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C. Navarre - *A small proteolipid is associated with the plasma membrane H^+ -ATPase of Saccharomyces cerevisiae.*

A. Goffeau - *New H^+ and Ca^{2+} -ATPase genes in the yeast Schizosaccharomyces pombe.*

Y. Eilam - *An intracellular (ATP + Mg^{2+})-dependent calcium pump within the yeast Schizosaccharomyces pombe, encoded by the gene cta3.*

A. Rodríguez-Navarro - *Kinetic and gene expression evidences do not support that PMR2 (ENA1) encodes a Ca-ATPase.*

METABOLIC REGULATIONS
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J.M. Peinado - *Regulation of glucose and maltose consumption in Candida utilis under repressed and depressed conditions.*

A. Boiteux - *Metabolic studies on synchronously dividing yeast cells. Energy metabolism during cellular division.*

M.A. Navas - *Futile cycling in Saccharomyces cerevisiae.*

S. Vissers - *Molecular characterization of the UGA43 gene encoding a negative factor controlling the expression of three permeases in Saccharomyces cerevisiae.*

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2. *Tuesday 17:*

SYMPORTERS
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R.A. Weusthuis - *Kinetics of in vivo maltose/proton symport in Saccharomyces cerevisiae.*

C.C.M. Van Leeuwen - *Maltose-proton co-transport in plasma membrane vesicles of Saccharomyces cerevisiae.*

A.A. Eddy - *Proton circulation through the cytosine-purine proton symport of Saccharomyces cerevisiae.*

M.R. Chevallier - *The purine-cytosine permease of S. cerevisiae: recent results on the relationships between structure and function.*

F. Cássio - *Transport of short-chain carboxylic acids in the yeast Candida utilis: evidence for a general permease.*

AMINO ACIDS AND INOSITOL TRANSPORTS
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H. Sychrová - *Uptake of lysine in thiosine-resistant mutants of Schizosaccharomyces pombe.*

B. Völker - *myo- inositol transport in Saccharomyces cerevisiae.*

General dicussion on membrane potential and internal pH.
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C.A. Michels - *Comparison of two high-affinity maltose permeases from Saccharomyces.*

G.F. Fuhrmann - *Inhibition of glucose transport in Saccharomyces cerevisiae by uranyl ions.*

M.C. Loureiro-Dias - *Physiology of the snf3 mutant of Saccharomyces cerevisiae in continuous culture.*

M. Höfer - *Hexose-transport-deficient mutant of Schizosaccharomyces pombe: phenotype and genetics.*

M. Côrte-Real - *Transport of glucose in a mutant of the yeast Hansenula anomala with potential interest for the biological deacidification of grape must.*

Chairman: C.A. Michels

T. Gonçalves - *Pitfalls in the measurement of glucose transport in Saccharomyces cerevisiae.*

I. Spencer-Martins - *Modes of lactose uptake in Kluyveromyces marxianus.*

M.A. Herweijer - *Do baker's yeast strains have different sugar transport characteristics as compared with laboratory strains?*

General discussion on sugar transports.
Chairman: G.F. Fuhrmann

3. Wednesday 18: *MODELS AND REGULATION*
Chairman: D. Fraenkel

L.A. Okorokov - *The change of activities of some vacuolar transport systems during the yeast growth.*

C. Wrede - *Misuse of nonlinear Eadie-Hofstee plots.*

J. Lenz - *Optimization of membrane transport models using evolutionary strategies.*

D. Kuschmitz - *Kinetic analysis of the plasma membrane H^+ ATPase of yeast: dependence on extracellular pH.*

J.M. Thevelein - *The glucose induced RAS-mediated cAMP signaling pathway in the yeast Saccharomyces cerevisiae.*

Final discussion and closing of the meeting. D. Fraenkel and A. Rodríguez-Navarro.

PREFACE

Rosario Lagunas

PREFACE

Rosario Lagunas. Instituto de Investigaciones Biomédicas del CSIC. Madrid. SPAIN

Ten years ago a small group of scientists working in the field of yeast transport and energetics decided to join once a year to discuss their results and projects. Their aim was to organize small meetings in this field in a very informal and provocative atmosphere. It was expected that, in this way, all participants, no matter how experienced they were, would feel inclined to express their ideas and criticisms. So "Small Meetings on Yeast Transport and Energetics" (SMYTE'S) started and proceeded since then.

Last year in Zvikovské Podhradí (Czechoslovakia) it was decided that, in 1991, the SMYTE would take place in Madrid and Alonso Rodriguez-Navarro and myself were in charge of its organization. The Fundación Juan March generously accepted to support the meeting including it in its program on International Meetings on Biology. All of those who know Andrés Gonzalez, of the Fundación Juan March, would readily understand that after this acceptance the organization of the meeting was for us indeed a very easy task.

The meeting was divided in 9 sessions that included 38 oral presentations covering most relevant areas of research in yeast ions channels, transport systems, and ATPases. In addition, 4 general discussions related with these topics were organized. The total number of participants was 55. Among them, 16 came from Spain and the rest were foreing guests mainly from Europe and a few of them from USA, Mexico and Israel.

In the name of all participants I would like to thank the Fundación Juan March for its invaluable support. It was the Fundación who made possible a meeting that will remain in the memory of all of us. I want also to thank the participants for their lively discussions and their generous exchange of information that so much contributed to the success of the 9th SMYTE.

FIRST SESSION
CATION TRANSPORT AND CHANNELS

A. PEÑA
R. LAGUNAS
H. P. BODE
A. BERTL
V. VACATA

A possible gene coding for K^+ transport in Kluyveromyces lactis

M. Miranda, A. Brunner, R. Coria and A. Peña. Dept. of Microbiology, Instituto de Fisiología Celular. Universidad Nacional Autónoma de México. Apartado 70-600. 04510 México D. F., México.

A mutant of Kluyveromyces lactis deficient in K^+ transport was isolated by selecting clones resistant to 20 uM ethidium bromide in agar plates (Brunner et al., Arch. Biochem. Biophys. 217: 30-36, 1986). Ethidium bromide is a competitive inhibitor of K^+ transport in S. cerevisiae (Peña and Ramírez; J. Membr. Biol. 22: 369-384, 1975). The study of the mutant showed a requirement of more that 10 mM KCl in the medium for growth. Mutant and wild type cells showed significant differences in the kinetic constants for Rb^+ transport. In the mutant, the Km for Rb^+ transport was 20 time higher than in the wild type cells (from 0.5 mM to 10.4 mM). The values of V_m were 8.35 and 1.46 nmol $(min.mg)^{-1}$, for the wild type and the mutant yeast, respectively.

The mutant was crossed with a wild type strain, and the diploids Kdm^-/Kdm^+ were grown in media with 1 mM potassium medium (LSK1). The growth of these diploids was similar to that of the diploids Kdm^+7Kdm^+, showing that the mutation is recessive.

A heterocygotic diploid was allowed to sporulate, and the dissection of the cross revealed a typical mendelian seggregation pattern for the phenotypes Kdm^- EB^R/Kdm^+ EB^S, indicating that the mutation is nuclear, and also that the resistance to ethidium bromide and the deficiency of potassium transport are provided by the same gene, or are in two genes seggregating together.

One recombinant Matα Kdm^- EB^R was selected and transformed with the TRK1-1 gene from Saccharomyces cerevisiae, kindly donated by prof. R. Gaber, and no complementation was found for the kdm mutation of K. lactis. A genome fragment from K. lactis complementing the trk1Δmutation of Saccharomyces cerevisiae did not complement the kdm mutation in K. lactis, indicating that the mutation does not correspond to the TRK1-1 locus. In this moment, we are transforming the mutant with a K. lactis bank, to obtain the fragment that complements the mutation of this strain.

This fragment may be involved in the codification for K^+ transport in K. lactis, since the phenotype of resistance to ethidium bromide seggregates together with that of deficient

potassium transport. It is also interesting that ethidium bromide, the agent used to select the mutants, behaves also as a competitive inhibitor of potassium transport in yeast (Peña and Ramírez, J. Membrane Biol. 22: 369-384, 1975). This mutation, besides, seems to occurr in a locus different to that of the TRK1-1 gene cloned and sequenced by Gaber et al. (Mol. Cel. Biol. 8: 2848-2859, 1988).

TURNOVER OF THE K^+ TRANSPORT SYSTEM IN Saccharomyces cerevisiae

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Most enzymes in Saccharomyces cerevisiae are fairly stable maintaining their catalytic activities for long periods under different metabolic conditions. Sugar transports behave differently in this respect since a rapid inactivation is observed upon inhibition of proteinisynthesis. This inactivation is an energy dependent process stimulated by fermentable substrates and is apparently due to proteolysis. To see whether a low stability is a pecularity of sugar transports or also affects to other carriers of the yeast plasma membrane we have investigated the stability of the K^+ transport system. To this end we have followed its activity upon inhibition of protein synthesis by addition of cycloheximide.

Activity of the K^+ transport system has been measured by following Rb^+ uptake using atomic absorption spectrophotometry. Addition of cycloheximide to glucose growing yeast produced a decrease in the Rb^+ uptake that followed first order kinetics indicating a half-lifefor this carrier of about 3.5 h. When instead of glucose ethanol was present as energy source the inactivation occurred at lower rate and, in this case, a half-life of about 15 h could be calculated.

The described decay in the Rb^+ uptake could be due to the turnover of the carrier as well as to a decrease in the plasma membrane potential due to a decrease in ATPase activity. We have checked this possibility using two mutants with reduced expression of plasma membrane ATPase. The results obtained strongly indicate that the rapid inactivation of the K^+ transport observed in the presence of glucose is not consequence of a decrease in the activity of ATPase but mainly due to the unstability of the carrier itself. However, much greater contribution of ATPase to the K^+ transport inactivation take place when, instead of glucose ethanol was present.

In conclusion, the results obtained in this work indicate that, similarly to sugar transport systems, K^+ transport system is less stable than the bulk of proteins of S. cerevisiae and that its unstability is increased by the presence of fermentable substrates.

Accumulation of strontium by vacuolar protein targeting mutants of Saccharomyces cerevisiae

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In addition to our studies about divalent metal cation accumulation in vacuolar mutants reported previously (1), we have now examined accumulation of strontium by these mutants. With respect to its chemical properties as well as its behaviour in biological systems strontium is very similar to calcium. The use of strontium instead of calcium, facilitating the application of certain methods, is therefore suitable for the investigation of cellular calcium transport and sequestration.
We found that both vacuolar mutants examined by us preferentially, vpt 16 and vpt 13, display decreased accumulation of strontium in comparison with the respective parental strains. vpt 16 is devoid of any intracellular structure resembling a vacuole, whereas vpt 13 has a morphologically normal vacuole, but is impaired in vacuolar acidification, most likely due to deficient assembly of the vacuolar proton pump subunits (2,3). In both mutants strontium accumulation was decreased to a similar extent. This was the case at external strontium concentrations between 10 µM and 5 mM. This demonstrates the function of the yeast vacuole as a strontium or calcium store as well as the essentiality of the proton gradient across the vacuolar membrane for this function.
Our results obtained with intact cells therefore support the current view about vacuolar calcium sequestration in yeast by a calcium proton antiporter, which is based mainly on the experiments by Ohsumi & Anraku with vacuolar membrane vesicles (4). However, the fact that decreased strontium accumulation by the vacuolar mutants was apparent even at low external strontium concentrations is somewhat in contrast to the rather low calcium affinity (K_m 100 µM total calcium), which has been reported for the vacuolar calcium proton antiporter by Ohsumi & Anraku. Our results imply that the yeast vacuole is permanently and predominantly operative as an intracellular calcium store in yeast.
A considerable strontium uptake capacity remains in the vacuolar mutants, amounting to between 41 and 84% of the parental strains capacity. Further studies will have to address the question whether this is due to cytoplasmic buffering or sequestration into another organelle, for example the endoplasmic reticulum.

1) H.-P.Bode et al. 1990, Proc. 8th Small Meeting on Yeast Transport & Energetics, p. 15-16.
2) L.M.Banta et al. 1988, J.Cell Biol. 107, 1369-1383.
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ION CHANNELS IN *SACCHAROMYCES CEREVISIAE*

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Ion channels have been studied on the single channel level in the plasma membrane and in the tonoplast of *Saccharomyces cerevisiae* by patch clamp techniques.

The prime ion channel in the plasma membrane is selective for K^+ over Cl^- and Na^+. In inside-out patches (200 mM K^+ in the bath, 50 mM K^+ in the pipette), the current-voltage curve of the open channel is sigmoid and follows the kinetics of a cyclic two-state model (i: inside, o: outside) with one pair of voltage-dependent, and one pair of voltage-independent rate constants (in 10^6 s^{-1}: $k_{io}=63 \cdot f^{0.5}$, $k_{oi}=17 \cdot f^{-0.5}$, $\kappa_{io}=40$ and $\kappa_{oi}=42$, f being $\exp(V_m e/kT)$).

The switching kinetics depend on the cytoplasmic calcium concentration ($[Ca^{++}]_c$) and on the transmembrane voltage (V_m) in a complex manner: At low membrane voltages (V_m near 0 mV) and low $[Ca^{++}]_c$, long closed periods (*gaps*: τ_g>100 ms) are separated by short bursts, which occupy a larger fraction of time as $[Ca^{++}]_c$ rises and V_m becomes more positive. On the other hand, there are short *blockages* (τ_b: some 10 ms) of the bursts which occur more often as $[Ca^{++}]_c$ and V_m rise. The bursts themselves consist of fast *interrupts* (τ_i<1ms) of the open channel current. Within the bursts, the interrupts become shorter (vanish) and the open-periods longer, as V_m approaches +100 mV. This switching within a burst is independent of $[Ca^{++}]_c$.

The entire kinetics can be described by a reaction scheme with one open state and the three closed states (gaps, blockages, and interrupts) in parallel, with their particular, V_m- and $[Ca^{++}]_c$-dependent, stability constants: $K_g=1.7 \cdot 10^{-4} \cdot [Ca^{++}]_c^{-1} \cdot f^1$, $K_b=2.8 \cdot 10^{-3} \cdot [Ca^{++}]_c^{-2} \cdot f^2$, and $K_i=2.8 \cdot f^1$.

With these kinetics and the open-state current-voltage relationship, the time-averaged currents as functions of $[Ca^{++}]_c$ and of V_m are predicted quantitatively. The experimental data fit the predictions.

The main type of ion channel in the yeast tonoplast is cation selective ($P_K:P_{Na}:P_{Ca}=1:1:0.2$) with an open-channel conductance of about 120 pS (in 100 mM KCl). Channel open probability is strongly calcium- and voltage-dependent, favoring cation release from the vacuole (only small currents into the vacuole). Channel activity shows marked "rundown" (within 15-60 min) resulting in an unphysiologically high (mM) Ca^{++} requirement. "Rundown" can be prevented by reducing agents (dithiothreitol, ß-mercaptoethanol or reduced gluthathion). These reducing agents shift the calciumrequirement in "aged" preparations to the more physiological micromolar range. Treatment of yeast vacuoles for 20-40 min with subnanomolar cytoplasmic Ca^{++} (10 mM EGTA, no Ca^{++} added) abolished channel activity, which could not be restored by high cytoplasmic Ca^{++} and reducing agents. Calmodulin, applied to the cytoplasmic side of the membrane, did reactivate the channels. Chloramine-T, shown to inactivate calmodulin by oxidation of its methionine residues, irreversibly inactivated yeast tonoplast cation channels as well. From these results we conclude that the cation channels from yeast tonoplast are calcium/calmodulin dependent. Reducing/oxidizing agends seem to change the Ca^{++} affinity of calmodulin (or of the channel protein itself). This type of cation channel may play a role in metabolic triggered release of calcium from the yeast vacuole.

Multiconductance Ionic Channels in the Plasma Membrane of the Yeast *Schizosaccharomyces pombe*

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[2]*University of California, Berkeley, Division of Biophysics and Cell Physiology, Berkeley, CA 94720, USA*

The major result of our patch-clamp studies of the yeast *Schizosaccharomyces pombe* is the discovery of a multiconductance, voltage-dependent potassium channel. This channel exhibits a maximum conductance of 153 pS and sublevels of conductance spaced roughly 30-50 pS apart (Fig. 1); the most frequent events, however, are transitions between the closed and the 153 pS conductance states with no residency in any of the conductance sublevels. The channel is not inhibited by tetraethylammonium; similarly, ATP and stretch have no effect. The channel is only mildly selective for potassium over sodium, lithium, and chloride (Table 1). The frequency of the channel occurrence in patches implies that, on the average, 35 channels of this kind are present in the plasma membrane of a single cell. The channel gates in the region of physiologically relevant voltages, being closed at hyperpolarizing and open at depolarizing voltages (Fig. 2). The gating characteristics of the channel imply an equivalent gating charge of 1.25. The existence of multiple conductance levels in this type of cationic channels might explain the wide variety of conductance sources reported in other works (1-4): some of these apparently different channels may be different states of one multiple conductance channel.

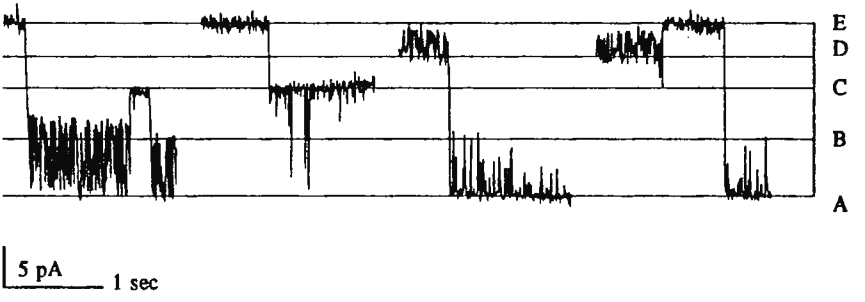

FIGURE 1. SUBLEVELS OF CONDUCTANCE OF THE 153 pS CHANNEL
Excerpts from current records of a single patch. In all traces the membrane was clamped at +90 mV. The coincidence in timing between large current jumps and the onset or cessation of current flicker in the new conductance levels indicates that all current events originate in one channel. The five conductance states are termed A through E, where A is the closed state and E is the fully open state. The current-voltage characteristics of individual conductance states (not shown) provide the values of conductance of the four open states: 43, 89, 128, and 153 pS. The patch was bathed in a standard clamp solution on both sides of the membrane: 140 mM KCl, 11 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES/TRIS, 591 mM sorbitol, pH 7.2, 0.90 Osm. The method for preparing protoplasts suitable for patch-clamping was reported earlier (5).

FIGURE 2. DWELL TIMES OF CONDUCTANCE SUBLEVELS OF THE 153 pS CHANNEL

Points are derived from sixteen 8 s (100 %) intervals following an applied voltage step from a holding potential of -80 mV to the various clamp potentials and take into account events of greater than ~50 ms duration. B through E correspond to the conductance states introduced in Fig. 1. In the physiologically relevant range of voltages, +30 mV and above, the highest degree of substate activity is observed, whereas bellow +30 mV and at negative potentials the channel resides in the fully open state E the whole of the time. In the physiologically relevant region, the fully open state E accounts for roughly half of the channel's activity. In general, the shape of the dwell time curve of state E corresponds to that of the expected gating activity of a potassium channel responsible for regulating the membrane potential across the yeast plasma membrane.

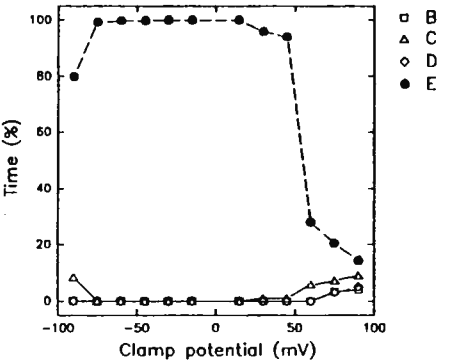

TABLE 1. PERMEABILITY RATIOS FOR MONOVALENT IONS IN THE 153 pS CHANNEL

	Li^+	Na^+	K^+	Cl^-
V_{rev} (mV)	-15.1	-10.2	0	-19.2
P_K/P_X	2.06	1.61	1	6.50

Permeability ratios P_K/P_X were derived from reversal potentials V_{rev} with 140 mM KCl inside the micropipette and 46.7 mM KCl in the bath solution (for the P_K/P_{Cl} ratio), and 140 mM KCl inside and 140 mM NaCl or LiCl in the bath solution (for the P_K/P_{Li} and P_K/P_{Na} ratios).

Acknowledgements. This work was supported by Deutsche Forschungsgemeinschaft Grant Ho555/13 to M.H., who was also a recipient of an academic fellowship by VW-Stiftung Hannover, and National Science Foundation Grant DCB-8904462 to H.L.

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SECOND SESSION
REGULATION OF CATION TRANSPORTS

J. RAMOS
R. L. BRANDAO
J. I. CASTRILLO

GLUCOSE ACTIVATION OF THE POTASSIUM UPTAKE SYSTEM AND PLASMA MEMBRANE H^+-ATPase OF YEAST. RELATIONSHIP WITH THE c AMP PATHWAY.

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Addition of glucose or other fermentable sugar to *Saccharomyces cerevisiae* cells induces a serie of regulatory processes. Many of them have been related to the transient increase in the cAMP level, which is one of the steps of the well known cAMP signalling pathway (1). Fermentable sugars activate the plasma membrane H -ATPase (2), and recently we reported that K^+uptake is also activated in these conditions (3). The possible relationship between the activation of the H -ATPase and the activation of K uptake with the cAMP pathway has been studied using different mutant strains.

We started this study making use of a fdp1 mutant strain, which lacks all the known glucose induced processes, is not able to grow on glucose, and depletes its ATP content upon addition of this or other fermentable sugar. In this mutant, the presence of glucose did not activate K uptake. An effect of difficult interpretation because fdp1 mutants do not grow on glucose. To address this uncertainty we used a fdp1 mutant strain harbouring a plasmid with the FPS1 supressor. The presence of the supressor allows the mutant to grow on glucose without recovering the glucose induced regulatory events (J. Thevelein, personal communication). In this strain, glucose did not activate K uptake.

Activation of the H -ATPase, and the increase in the cAMP level produced by glucose are phenomena completely dependent on the presence of a kinase able to phosphorylate the sugar (J. Thevelein, personal communication). Our results using different sugar kinase mutants suggest that full activation of the K uptake system by a sugar also requires the presence of a functional sugar kinase.

In contrast with the results obtained with the fdp1 mutant and sugar kinase mutants, mutations in genes downstream in the cAMP pathway did not affect the activation of K uptake. Thus, in a cdc25 ts mutant, which has been described not to be able to activate the H -ATPase by glucose, we found that activation of K^+ uptake by glucose occurs similarly at low and at restrictive temperatures. Besides, (i) mutants in one or two RAS genes, (ii) mutants failing to show the transient increase in cAMP induced by glucose but with a normal basal level of cAMP, and (iii) mutants in the different TPK genes did not show any difference with the behaviour of wild type strains, increasing the rate of K^+ uptake in the presence of glucose.

Present results support the idea that glucose regulated pathways independent of the cAMP pathway occur in yeast.

Recently, it has been suggested that glucose regulated pathway of cAMP-synthesis and the process of H^+-ATPase activation can have a common initiation point, but they separate later. Apparently, the pathway for glucose activation of K^+ uptake also shares some steps with the cAMP pathway, but they separate very early.

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Mechanism of glucose-induced activation of plasma membrane H^+-ATPase in cells of the yeast *Saccharomyces cerevisiae* .

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Addition of glucose, related sugars or protonophores to derepressed cells of the yeast *Saccharomyces cerevisiae* causes within a few minutes a three- to fourfold activation of the plasma membrane H^+-ATPase. These conditions are known to cause rapid incresed in the cAMP level. In yeast strains carrying temperature-sensitive mutations in gene products required for cAMP synthesis, incubation at restrictive temperature reduced the extent of H^+-ATPase activation to variable degrees. Incubation of wild type strains however at such temperatures also cause variable reductions of H^+-ATPase activation. Yeast strains which are specifically deficient in the glucose-induced cAMP increase (but not in basal cAMP synthesis) still showed plasma membrane H^+-ATPase activation. Yeast mutants with widely divergent activity levels of cAMP-dependent protein kinase displayed very similar levels of activation of the plasma membrane H^+-ATPase. Recently, it was shown that the cAMP-protein kinase A signaling pathway probally possesses a glucose-repressible protein. Addition of glucose to cells of cat1 and cat3 mutants, which are deficient in derepression of glucose-repressible proteins, do not induce a cAMP signal, but provoke a typical H^+-ATPase activation. These results tend to exclude the cAMP-protein kinase A signaling pathway as the mediator of the glucose effect on the H^+-ATPase.

Experiments with yeast strains carrying point or deletion mutations in the genes coding for the sugar phosphorylating enzymes hexokinase PI and PII and glucokinase showed that activation of the H^+-ATPase with glucose or fructose was completely dependent on the presence of a kinase able to phosphorylate the sugar. The yeast *fdp1* mutant, which is deficient in glucose-induced activation of the cAMP signaling pathway is also deficient in glucose-induced activation of plasma membrane H^+-ATPase. These data suggest that the glucose-induced activation pathways of cAMP-synthesis and H^+-ATPase have a common initiation point. On the other hand, addition of protein kinase C activators (oleic and arachidonic acids or diacylglycerol) also causes H^+-ATPase activation. This result points to the possible participation of protein-kinase C in the activation mechanism.

THE CHEMOSTAT. A VALUABLE TOOL FOR THE *IN VIVO* DETERMINATION OF THE SPECIFIC RATE OF PROTON PRODUCTION (qH+) FROM YEAST CELLS.

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The mechanisms of interaction between the microbial cell and its environment in fermentation systems are subject of continuous investigation (1). In this context, the mechanism of formation of transmembrane pH gradient (ΔpH) constitutes a factor of particular importance. Transmembrane pH gradient is involved in the formation of electrochemical proton gradient across cell membranes which provides the source of energy for ATP synthesis and active transport (2).

Different approaches have been carried out in order to measure the rate of proton extrusion/consumption of cells: Spectrophotometric methods using acid/base indicators and fluorophores have been used to determine the proton pumping in vesicles and harvested cells ("*in situ*" methods) (3-6). On line measurement of acid/alkali consumed in a pH-controlled reactor has been also widely used (6-10). However, aspects such as the contribution of other molecules on H+ concentration, or the buffering capacity of the medium have been rarely considered. In many cases studies have been reduced to establishing empirical relations for use in control of fermentation process.

The chemostat allows the management of a population of microrganisms in a defined and constant environment at a precise specific growth rate, being a suitable system for *in vivo* studies. This system has been therefore selected to develop a rigorous method to calculate the specific rate of proton production qH+ (meq H+ g-1 h-1) at a defined metabolic state.

The method comprises three independent steps: 1) On line determination of flux of acid/alkali required to maintain constant pH of chemostat at a defined steady state. 2) Qualitative and quantitative determination of the contribution of other molecules on H+ concentration, and 3) Evaluation of buffering capacity of the medium at the defined conditions. The combined execution of these three steps allows, after application of mass balance for H+, the determination of the specific rate of proton production (qH+).

The method has been applied to chemostat cultures of *Kluyveromyces marxianus* growing in whey. The obtained results show qH+ increases proportionally with increasing growth rates, with values passing from negative (net proton consumption) to positive (net proton extrusion) throughout the range of dilution rates. Analysis of this behaviour and the possible relationships between proton interchange processes and specific biochemical functions will be presented.

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THIRD SESSION
ATPases

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C. NAVARRE
A. GOFFEAU
Y. EILAM
A. RODRÍGUEZ-NAVARRO

Plasma membrane H^+-ATPase and secondary active transports in different yeasts

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Several strains of *Saccharomyces cerevisiae*, including the *pma1*-105 mutant with defective major H^+-ATPase of the plasma membrane, as well as *Rhodotorula gracilis* and *Lodderomyces elongisporus* were grown to early stationary phase and the following properties were estimated: membrane potential (with tetraphenylphosphonium chloride), ΔpH (glass electrode for pH_{out}, dual-wavelength fluorescence of fluorescein for pH_{in}), ATP-hydrolyzing activity in membrane fragments with suppressed F-ATPase (azide) and V-ATPase (nitrate), ATPase-associated acidification of the external medium, and initial rates of uptake of 14 amino acids over a concentration range from 1 μM to 10 mM and (in *R. gracilis* and *L. elongisporus*) of 6-deoxy-D-glucose. Various inhibitors/activators of the P-type ATPase were tested, such as D_2O, diethylstilbestrol, vanadate, suloctidil, erythrosine B, miconazole, cupric sulfate and fusicoccin. There was a striking similarity between the effects of the inhibitors on ATPase activity (especially that reflected in the acidification potency) and the rates of uptake of the amino acids and 6-deoxy-D-glucose. There was virtually no such similarity between the calculated protonmotive force and the rates of uptake. The inhibitory effects were always more pronounced toward the uptake of acidic than of neutral and especially basic amino acids - mean inhibition by, e.g., D_2O was 72, 46 and 29 %, respectively. This is taken as evidence of a topological relation between the H^+-ATPase and the amino acid carriers which, apparently, sense the local concentration of H^+ produced by the ATPase within or at the membrane, rather than the "macroscopic" protonmotive force.

A small proteolipid is associated with the plasma membrane H^+-ATPase of Saccharomyces cerevisiae.

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The plasma membrane of yeast contains a well-characterized ATPase that pumps protons out of the cell, energizing the membrane for nutrient uptake. The yeast H^+-ATPase is structurally and functionally related to the Na^+/K^+, Ca^{2+} and H^+/K^+-ATPases of animal cell membranes. Upon classical Laemmli gel electrophoresis, the purified yeast H^+-ATPase displays a highly enriched 100 KDa band which corresponds to the single catalytic subunit. It has been reported however that the *Schizosaccharomyces pombe* preparation contains, in addition to the 100 KDa band, a component moving slightly ahead of the tracking dye.

We have examined the possibility that the fungal H^+-ATPase might possess a small additional subunit. Using electrophoresis conditions adopted to the separation of low molecular weight proteins, the purified H^+-ATPase of *Saccharomyces cerevisiae* displays two Coomassie-stained bands of low molecular weight (7,5 KDa and 4 KDa). These two polypeptides can be isolated by a chloroform/methanol extraction establishing their proteolipidic nature. A treatment with ether allows to separate the two bands; the 4 KDa component is soluble while the 7,5 KDa compound precipitates. The amino acid composition and the NH_2-terminal sequence were identical for the two components, suggesting that the extracted proteolipid exists as a dimer and a monomer. The microsequencing of the purified protein revealed the existence of a small polypeptide of 38 amino acids with a calculated molecular mass of 4250 Da. The corresponding gene was isolated by hybridization to an oligonucleotide probe derived from a portion of the amino acid sequence. The gene sequencing confirmed the amino acid sequence with the exception of two additional amino terminal residues. The amino acid sequence is predicted to contain a NH_2-terminus transmembrane segment followed by a very basic hydrophilic domain.

New H^+ and Ca^{2+}- ATPase genes in the yeast *Schizosaccharomyces pombe.*

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H^+-ATPase

The fungal plasma membrane contains a well-characterized ATPase that pumps protons out of the cell, energizing the membrane for nutrient uptake. In *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa*, the plasma membrane H^+-ATPase is encoded by the *PMA1* gene which is essential to cell growth. The existence of a second H^+-ATPase in *S. cerevisiae*has been inferred from the isolation of *PMA2*, a gene highly homologous to *PMA1* (Schlesser *et al.* , J. Biol. Chem. **263**, 19430-19487, 1988). The physiological function of the *PMA2* gene is unknown, however, since a null allele confers no obvious phenotype.

We have isolated a second H^+-ATPase gene in *S. pombe* and report its sequence. This gene, referred to as *pma2* codes for a polypeptide having a predicted M_r = 110,126 and which is 79 % identical to the plasma membrane H^+-ATPase encoded by the *pma1* gene. The *S. pombe pma2*gene, unlike *pma1*, is weakly expressed and not essential to mitotic growth. By constructing yeast strains in which the chromosomal *pma2* gene is under control of the alcool dehydrogenase promoter from *S. pombe*, it has been possible to identify the overproduced ATPase protein in plasma membranes, via formation of a phosphoenzyme. In a *pma1-1* mutant strain whose ATPase activity is insensitive to vanadate, the overexpressed *pma2* gene restores vanadate-sensitivity. it also rescues a *pma1* null mutant from lethality. These results demonstrate that the two H^+-ATPases are functionally interchangeable *in vivo* but differently expressed.

Ca^{2+}-ATPase

Another new P-type ATPase gene, *cta3*, has been identified in *Schizosaccharomyces pombe*. The deduced amino acid sequence presents a 45 % identity with the *Saccharomyces cerevisiae* putative Ca^{2+}-ATPase encoded by the *PMR2* gene. The cta3 protein contains 7 out of the 8 amino acid residues involved in high affinity Ca^{2+} binding in the sarcoplasmic reticulum Ca^{2+}-ATPase from muscles. It also contains a region similar to the phospholamban-binding domain that characterizes this Ca^{2+} pump. A null mutation of *cta3* modifies calcium homeostasis and cellular transports as described by Y. Eilam and collaborators in this meeting. The sequence analysis and the physiological results strongly support the conclusion that the *cta3* gene encodes a Ca^{3+}-ATPase, located in intracellular membranes.

AN INTRACELLULAR (ATP + Mg^{2+})-DEPENDENT CALCIUM PUMP WITHIN THE YEAST SCHIZOSACCHAROMYCES POMBE, ENCODED BY THE GENE cta3

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Evidence has been accumulated that in yeast cells, cytosolic Ca^{2+} ($[Ca^{2+}]_i$)-homeostasis is maintained at levels comparable to the levels in animal and plant cells but the mechanisms regulating $[Ca^{2+}]_i$ in yeast have not yet been identified. To investigate Ca^{2+} accumulation into intracellular Ca^{2+} storing organelles we have permeabilized the plasma membranes of S. pombe cells with nystatin and measured ATP-dependent Ca^{2+} uptake. Since yeast vacuoles take up Ca^{2+} by Ca^{2+}/H^+ exchangers which are linked to ATP utilization through $\Delta\tilde{\mu}_H$, established by the V-type H^+ ATPase, Ca^{2+} uptake was measured in the presence of KNO_3, an inhibitor of V-type ATPases, and CCCP, a protonophore which excludes any H^+-coupled Ca^{2+} transport. ATP-dependent Ca^{2+} accumulation into non-vacuolar Ca^{2+} storing organelles was detected. This Ca^{2+} uptake activity was maximal at pH 6 and could be inhibited by vanadate, the inhibitor of P-type ATPases. We have recently reported the identification of a novel gene in S. pombe, cta3. The deduced amino acid sequence suggested that this gene encodes a P-type ATPase and the physiological results suggested an intracellular Ca^{2+} pump (Ghislain et al., J. Biol. Chem. 265, 18400-18407, 1990). At present we have found that the strain MG4, harboring the null mutation in cta3 had a markedly reduced level of ATP-dependent Ca^{2+} uptake into non-vacuolar intracellular storing organelles. This observation indicates that cta3 encodes an intracellular Ca^{2+} pump. The residual ATP-dependent Ca^{2+} uptake in MG4 strain indicates the presence of a second Ca^{2+} ATPase encoded by a different gene.

KINETIC AND GENE EXPRESSION EVIDENCES DO NOT SUPPORT THAT PMR2 (ENA1) ENCODES A Ca-ATPase

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Two years ago in the VIISMYTE we reported the cloning of gene ENA1, which was able to confer Li tolerance to a low Li-efflux yeast strain. In subsequent work we determined the sequence on ENA1 and proximate regions, finding that ENA1 encoded a P-ATPase, and that three copies of the gene were in tandem.

The same ENA1 gene has also been cloned by its homology with PMA1, the gene encoding the H-pump ATPase of yeast. This gene was named PMR2, and it was proposed to encode a Ca-ATPase, by the homology of its predicted amino acid sequence with the sequences of other Ca-ATPases [H.K. Rudolph, et al 1989, Cell, 58:133-145]. However, disruption of ENA1 and the copies in tandem with it produces a phenotype of Na and Li sensitivity, and decreases severely Na and Li effluxes. Defects not immediately related to the lack of a Ca-ATPase.

Two possible hypotheses could explain the dependence of Na and Li effluxes from a Ca-ATPase, and conciliate physiological evidences and homology predictions: (i) Na and Li effluxes are coupled to Ca influx, and a Ca-ATPase mediates Ca efflux, and (ii) Na, Li and Ca effluxes are mediated by the same ATPase.

The first possibility was investigated by studying the kinetics of Li efflux in buffers with and without Ca, and with EDTA and EGTA at pH 8.5, finding that the concentration of Ca did not affect significantly Li efflux. To investigate whether a Ca-ATPase transports Na and Li, we studied the expression of ENA1 since the expression of this gene is not constitutive. Na, Li, or even K, specially at high pH induces the expression of ENA1, but we have not been able to express it with Ca.

All the evidence we have found so far supports that ENA1 (PMR2) encodes a Na-ATPase. No evidence has been found suggesting that it encodes a Ca-ATPase.

FOURTH SESSION
METABOLIC REGULATIONS

J. M. PEINADO
A. BOITEUX
M. A. NAVAS
S. VISSERS

"Regulation of glucose and maltose consumption in Candida utilis under repressed and derepressed conditions"

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We have studied the consumption of glucose and maltose by Candida utilis cells, growing in continuous cultures on a synthetic medium with both sugars as carbon and energy source. The steady-state specific activity of the two permeases for glucose and those of maltose permease and maltase were also determined in cells that had grown at each dilution rate (D) for at least 5 residence times.

At low D values, with steady-state glucose concentrations below 100 mg/l, both sugars were consumed simultaneously. There was no repression, and maltase specific activity maintained a constant level at any D value. However, the level of maltose permease decreased as D increased, most probably due to glucose inactivation. The specific activity of the glucose permeases also changed: The glucose-proton symport increased with D, as was expected of an inducible transport system. The glucose and maltose consumption rates, measured "in vivo" in the fermenter, increased linearly with D and so did the total metabolic flux. The individual rate for each sugar depended on its concentration in the inflowing medium and not on the kinetic parameters of the transport systems.

These results indicate that sugars transport is not limiting growth in these conditions, in contrast with what happened in previous experiments with Saccharomyces cerevisiae growing on maltose. In this last case, maltose permease did limit growth.

METABOLIC STUDIES ON SYNCHRONOUSLY DIVIDING YEAST CELLS ENERGY METABOLISM DURING CELLULAR DIVISION

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Modern analytical techniques have come up with methods to study a variety of cellular events directly in single cells. For certain studies, however, current analytical methods require the use of large cell populations. We have used substrate limited synchronized continuous cultures of S. cerevisiae (ATCC 9080) to study the energy metabolism of the yeast cell during cellular division. It is the advantage of continuous cultures that cells can be maintained in different metabolic states - the disadvantage is the need for working under sterile conditions.

By proper synchronization our culture shows regular cycles of simultaneous cellular division (> 50 % of the population) which can be maintained indefinitely. About one hour before any sign of budding can be observed in the microscope, a strong pulse of CO_2 evolution can be detected by electrochemical measurement and titration. Metabolic analysis reveals an "exploding" glycolytic flux induced by activation of the enzyme phosphofructokinase, leading to the depletion of the cellular storage carbohydrate, trehalose, within just 20 minutes. During this glycolytic pulse the uptake of glucose from the medium is blocked completely. Simultaneously, the produced ethanol is oxidized slowly to acetate and further to CO_2 and water within the next 90 minutes as shown by analysis of metabolites, oxygen saturation and titration.

Inspection of mass action ratios indicates that the glycolytic flux is modulated by a few key enzymes. Whereas most of the enzymes like phosphoglucomutase, trehalase, hexokinase and phosphoglucose isomerase maintain their steady state or equilibrium conditions even at highest flux rates, we calculate deviations up to a factor of 10 for phosphofructokinase, aldolase, adenylate kinase and pyruvate decarboxylase. It is interesting to note that exactly these enzymes generate the cyclic flux of the well known "glycolytic oscillations".

The questions raised are: When is the cell ready for mitosis to take place? A necessary disposition for cellular division under glucose limited growth conditions is certainly the availability of sufficient storage carbohydrate to carry out the formation of a daughter cell. Can the cytosolic concentration of trehalose (glucose) possibly meet a maturity threshold? And what, actually, is the trigger for the starting glycolytic pulse? From substrate pulse experiments it seems obvious that the trigger signal is generated on the plasma membrane or in the cytosolic space itself.

FUTILE CYCLING IN *SACCHAROMYCES CEREVISIAE.*

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When *S.cerevisiae* is growing on glucose the gluconeogenic enzymes fructose 1,6 bisphosphatase (FbPase) and phosphoenol- pyruvate carboxykinase (PEPCK) are repressed. Moreover when glucose is added to a yeast with derepressed gluconeogenic enzymes these are rapidly inactivated. It would seem therefore that the cell should exert a strict control on the amount of the gluconeogenic enzymes to avoid futile cycles that would waste ATP at the level of the pair of antagonistic enzymes phosphofructokinase-FbPase, pyruvate kinase-PEPCK.

To test this idea we have constructed yeast strains in which the gluconeogenic enzymes would escape this control. In these strains the expression of the genes FBP1 and PCK1 in multicopy plasmids is no longer repressed by glucose. The strains are viable on glucose and have high levels of FbPase and/or PEPCK. Their generation time on glucose is only slightly increased (about 20% for the transformant containing the two unregulated enzymes) and their levels of adenine nucleotides and hexose phosphates are the same as those of the untransformed yeast.

It appears therefore that the presence of the gluconeogenic enzymes in a yeast growing on glucose has only a marginal effect, although the limited decrease in the growth rate would still be enough to give a selective advantage to a strain with regulated enzymes.

We can see at least two explanations, which are not mutually exclusive, for the results obtained:

-During growth on glucose, the gluconeogenic enzymes are present but are not operative due to some mechanism for regulation of their activity.

-Glycolysis produces more ATP than needed and therefore although futile cycling increases the ATP utilization this has not excessive consequences for the cells.

Molecular characterization of the *UGA43* gene encoding a negative factor controlling the expression of three permeases in *Saccharomyces cerevisiae*

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The use of 4-aminobutyrate (GABA), urea or allantoin as sole nitrogen source by *Saccharomyces cerevisiae* involves three inducible permeases. Synthesis of the GABA-specific permease (UGA4) is induced by GABA. Allophanate, an urea- degradation intermediate, induces the synthesis of the urea (DURP) and allantoin permeases (DAL4).

GABA-dependent induction requires the product of the *UGA3* gene acting as a positive transcriptional factor. Allophanate induction requires the product of the *DURM* gene, also acting as a positive factor. In addition, a second positive factor encoded by the *UGA35* (*DURL/DAL81*) gene is required for both processes.

Genetic evidence suggests that UGA35 in association with UGA3 or DURM, respectively, activates transcription by counteracting the negative effect exerted by the *UGA43* (*DAL80*) gene-product. The genes encoding the three positive factors have been previously sequenced and analysed (1). In order to complete this first step in the molecular characterization of these induction mechanisms, the *UGA43* gene has now been cloned and sequenced. The deduced polypeptide is 269 aa long. A putative DNA-binding domain (cystein-rich) and a putative oligomerization domain (leucine zipper) were found respectively near the NH_2 and COOH extremities. Mutations were located in these two domains in *uga43* minus mutants.

A cystein-rich domain very similar to the UGA43 one is also present in three fungal activators, Nit2 (*N. crassa*), AreA (*A. nidulans*), GLN3 (yeast) and in the GATA-1 gene family (vertebrates). Northern hybridization experiments show that the UGA43 RNA is largely present after growth on M. proline. There were no significant variations due to the presence of GABA or urea in the medium. Expression is subject to nitrogen catabolite repression. Indeed, when ammonium ions were present in the growth medium the UGA43 RNA level decreased drastically. It was also observed that in a *uga43* minus mutant grown on M. proline, the UGA43 RNA-level was much higher than in the wild-type strain on the same medium. Hence the *UGA43* gene is probably auto-repressible.

These results and those previously obtained in the molecular study of the positive factors are in full agreement with our model based on classical genetic studies.

(1) See Coornaert, D., Vissers, S., André, B. Gene, 97 (1991) 163-171 and references therein.

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FIFTH SESSION
SYMPORTERS

R. A. WEUSTHUIS
C. C. M. VAN LEEUWEN
A. A. EDDY
M. R. CHEVALLIER
F. CASSIO

Kinetics of *in vivo* maltose/proton symport in *Saccharomyces cerevisiae*

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Maltose transport in *Saccharomyces cerevisiae* is known to occur via proton symport. Since protons are a co-substrate for the maltose permease, maltose transport must be affected by proton concentrations. These effects have been investigated in three ways: kinetics of growth, kinetics of transport, and ^{31}P-NMR. Since differences in pH not only can affect transport but metabolism as well, glucose was used as a control when appropriate (glucose is transported by facilitated diffusion).
For the transport studies and the NMR experiments continuous culture cells were used, since they exhibit higher V_{max} values and can be grown carbon-limited on maltose and glucose at the same time.

Growth kinetics

Saccharomyces cerevisiae was grown in batch culture with maltose or glucose as carbon source at pH 5.0. The effect of a shift in pH to 7.0 on the growth rate was determined (Table 1).
The growth rate on glucose was hardly affected, the growth rate on maltose diminished.

Table 1. Effect of pH on growth rates (h^{-1}) of *Saccharomyces cerevisiae* with maltose or glucose as carbon source.

	Growth rate (h^{-1})	
Carbon source	pH 5.0	pH 7.0
maltose	0.37	0.21
glucose	0.37	0.33

Kinetics of maltose permease using U-^{14}C maltose

The kinetics of maltose uptake of *S. cerevisiae*, grown at pH 5.0, at different pH values has been studied using U-^{14}C maltose. The V_{max} for maltose uptake varied with pH (Figure 1). In a pH range from 3.0 to 7.0 the K_m for maltose uptake varied between 2 and 4 mM. Between pH 7.0 and pH 4.5 maltose permease exhibited Michaelis-Menten kinetics towards protons with an apparent pK_H of 5.7.

Comparison of kinetics of maltose and proton uptake

The kinetics of maltose and proton uptake at pH 5.0 were measured. The K_m values were approximately 3.0 and the maltose/proton stoichiometry was determined to be 0.8. A value of 1.0 was determined using cytochrome *c* oxidase-containing vesicles. (See C.C.M. van Leeuwen *et al.*, this book).

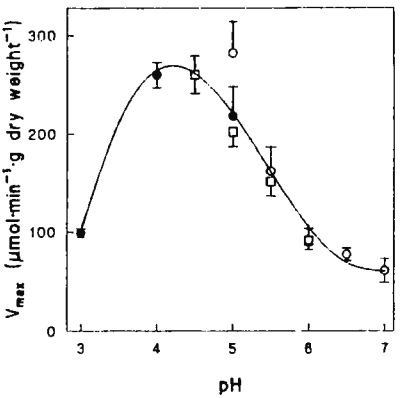

Figure 1. Effect of pH on the maximal uptake rate (μmol.min^{-1}.g^{-1}) of ^{14}C maltose by *Saccharomyces cerevisiae*. The different symbols refer to cell suspensions from independent steady states.

Effect of maltose uptake on cytosolic pH

Since one proton is co-transported with maltose, maltose transport will change the intracellular pH. The changes in cytosolic pH upon addition of maltose were determined

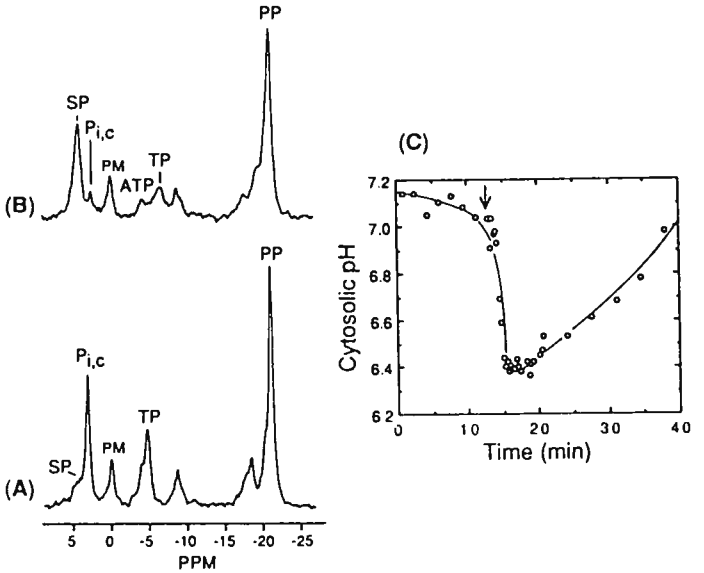

Figure 2. Consequences of maltose uptake by *Saccharomyces cerevisiae* for the ^{31}P-NMR spectra and on cytosolic pH. Spectra were obtained at 121.5 MHz and are the result of 1000 scans acquired in 3.5 min, either before (A) or after (B) the addition of maltose at an external pH of 5.0. Peak assignments: SP, sugar phosphates; $P_{i,c}$, cytosolic pH; PM, cell wall phosphomannan; ATP, γ-phosphate in ATP; TP and PP, terminal and middle phosphates in polyphosphate, respectively. Fig. 2C depicts the cytosolic pH as determined from the chemical shift of cytosolic P_i as described previously (Nicolay *et al.* 1982). Arrow indicates the point of addition of 60 mM maltose directly into the suspension while in the NMR instrument.

using ^{31}P-NMR. By comparing the peak positions of cell wall phosphorus (independent of cytosolic pH) and inorganic phosphate (dependent on cytosolic pH) the cytosolic pH can be determined (figure 2). Double-molar amounts of glucose were used as control.

Addition of maltose at an extracellular pH of 5.0 gave rise to an immediate decrease of cytosolic pH from 7.1 to 6.4, followed by a slow recuperation. Addition of glucose led to a drop in pH from 7.1 to 6.8, followed by an increase in pH to 7.5 and a slow decrease to 7.1. Addition of glucose at an extracellular pH of 7.0 gave the same results. Addition of maltose at pH 7.0, on the other hand, resulted in a much less pronounced effect, consistent with kinetical properties of maltose permease.

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Maltose-proton co-transport in plasma membrane vesicles of Saccharomyces cerevisiae

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Maltose proton co-transport was studied in plasma membrane vesicles of *S.cerevisiae*. Plasma membranes were isolated from cells with a constant high maltose transport capacity by using maltose-limited continuous cultures. In order to study uphill maltose transport, the plasma membrane vesicles were fused with cytochrome c oxidase proteoliposomes. The method yielded hybrid proteoliposomes of which 30 -35 % contained an active maltose permease. Energization with a suitable electron-donor system resulted in the generation of a proton-motive force (PMF), inside negative and alkaline. Moreover, maltose accumulation was observed. Both the initial influx velocity and accumulation ratio decreased with increasing pH. Determination of the kinetic constants at pH 5.5 and 6.7 revealed that this decrease in velocity is a consequence of the V_{max}, decreasing dramatically at higher pH values, the Km value remaining virtually constant (Km$\sim$10 mM). Uptake of tracer concentration of maltose (15 µM) at pH 5.5 showed a maximal accumulation ratio of 33. Addition of the ionophores valinomycin (abolishing the $\Delta\psi$), or nigericin (abolishing the ΔpH) decreased the accumulation ratio, while simultaneous addition of ionophores at steady state accumulation level resulted in a rapid efflux of accumulated maltose, proving that maltose proton symport is both $\Delta\psi$ and ΔpH dependent. From the chemical gradient ($\Delta\mu_{solute}$), calculated from the steady state accumulation ratio, and the PMF (Δ p), determined when maximum accumulation was achieved, the stoichiometry (n_{app}) could be calculated, according to the equation;

$$n_{app} = \mu_{solute} / \Delta p$$

It is concluded that the average stoichiometry of the maltose proton co-transport is 1, consistent with the results obtained with intact cells. (See R.A. Weusthuis *et al.*, this book)

Table 1. Relation between p and maltose uptake.

Addition	Δ p (mV)	$\Delta\mu_{solute}$ (mV)	n_{app}
none	115.5	91.4	0.8
37 nM nigericin	66.9	88.6	1.3
100 nM valinomycin	77.1	67.7	0.9

PROTON CIRCULATION THROUGH THE CYTOSINE-PURINE PROTON SYMPORT OF SACCHAROMYCES CEREVISIAE

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The cytosine-purine transport protein gene (FCY2) has been cloned by Chevallier on a multicopy plasmid (pNCII4-9) (Schmidt et al., Proc. Nat. Acad. Sci. 81, 6276, 1984) and corresponds to a peptide of apparent molecular mass of 45 kDa (Chirco et al., Eur. J. Biochem. 194, 293, 1990). Our previous studies with a voltage sensitive dye indicate that symport of protons through the carrier is an electrogenic process in yeast strain NC233-10b (fcy1 fcy2 leu2) rendered prototrophic by pNCII4-9 [LEU2 FCY2] (Hopkins et al., FEMS Microbiol. Lett. 49, 173, 1988). Proton absorption is thus probably not directly coupled to the concomitant efflux of K^+ ions that occurs when the proton movements are assayed.

We have now studied both the proton absorption and the membrane depolarization that occur in the presence of each of twenty potential substrates. Of nine substitutions at the 5 position in cytosine, the well known 5-fluoro derivative has shown unexpectedly complex behaviour. Cytosine itself is absorbed along with 1 equivalent of protons per mole of cytosine in the above yeast strain, whereas 4-6 proton equivalents accompany 5-fluorocytosine. Comparison of the above behaviour with that of NC233-10b [LEU2] and FL442-2D (fcy1 FCY2) (Hopkins et. al., loc. cit.) shows that the superstoichiometry exhibited by fluorocytosine is not due to the presence of a leak pathway for 5-fluorocytosine outside the carrier, nor is it a consequence of overexpression of the carrier.

We suggest that this interesting phenomenon arises because fluorocytosine is an imperfect substrate of the carrier. We anticipate that point mutations in FCY2 may lead to smaller values of the proton stoichiometry for 5-fluorocytosine.

The behaviour of 5-fluorocytosine is unexpected because the concentration of this substrate from dilute solutions proceeded to very roughly the same extent as that of cytosine, forming gradients of about 12.5 kJ mol^{-1} in both instances. This suggests that the excess protons absorbed along with the fluoro-derivative do not perform useful work during their passage through the symport.

The purine-cytosine permease of *S.cerevisiae* : recent results on the relationships between structure and function

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As already reported, making use of the variety of substrates transported by the purine cytosine permease (PCP), "Km" mutants were isolated by several *in vivo* selection procedures.So far, seven alleles of the *FCY2* gene were cloned and sequenced.

The first mutant sequenced, *fcy2-21*, had three aminoacids changed in the primary structure of the PCP (533 aa, 58 kD) : Ile371->Val, Ile375-> Val and Asn377->Gly. Detailed analysis of Km and Kd (binding) were made on strains carrying this allele and on wild type. The mutant shows an increase in Km for all ligands except adenine and the Kd were dramatically increased for all ligands, with a correlation to the variations in Km of uptake for cytosine and hypoxanthine. Therefore we conclude that the modification in Km in this mutant are merely due to a modification of the binding ability of the PCP for its ligands.

Since we found three aminoacid changed in the *fcy2-21* allele it was important to determine the contribution of the individual changes to the observed phenotype. By use of *in vitro* site directed mutagenesis we constructed two new mutants one with Asn377->Gly and the second with Ile371, Ile375->Val. Uptake measurements were made which showed that the mutation Asn377->Gly lead to a decrease in affinity for cytosine without noticeable change in Vmax, whereas the mutations Ile371, Ile375->Val only slightly affect the affinity for cytosine and seem to bring along an increase in Vmax for cytosine uptake of 2-3 which is also found on the original *fcy2-21* allele. These results wich must be strengthened by more detailed measurements suggest different roles for the different positions.

Among the other mutants, we found three identical alleles (*fcy2-22, fcy2-23 ans fcy2-24*)with the same multiple changes displayed by *fcy2-21* and three additional mutations. Three alleles had single changes : *fcy2-10* with Asn377->Leu, *fcy2-20* with Asn374->Ileu and *fcy2-5* with Gly486->Arg.

Therefore our results point out to the segment **371-377** as being involved in determining the transport ability of the PCP, but in addition the position 486 plays some role, since a single change at this position was found in the mutant *fcy2-5* . This mutant shows a decrease in the affinity and significant reduction in Vmax for cytosine.

According to our actual model which will be discussed, all these mutations are located on cell surface facing regions and not in transmembrane segments as was found for the lactose permease.

The identical multiple mutations we found in four independently obtained alleles could be the result of a genic conversion, which would involve the existence of another gene having at least one high homology region with *FCY2*, hypothetical gene which we intend to search with the help of probes coming from mutated alleles.

TRANSPORT OF SHORT-CHAIN CARBOXYLIC ACIDS IN THE YEAST *Candida utilis* : EVIDENCE FOR A GENERAL PERMEASE

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Growth of the yeast *Candida utilis* in a medium with mono, di, or tricarboxylic acids as carbon and energy sources induced two transport systems for these acids presumably a proton symport and a facilitated diffusion for the charged and the undissociated form(s) of the acids, respectively. Both systems could be observed simultaneously when the transport was measured with labelled carboxylic acid at pH 3.0. The two transport systems exhibited different substrate specifities. The high affinity system was specific for the respective carboxylic acid family. With respect to the low affinity transport system evidence was available that the system might be a general carboxylic acid permease which accepted mono, di or tricarboxylic acids. Both systems were inducible and were subjected to glucose repression. The induction was not dependent on the relative concentration of anionic form(s) and of undissociated carboxylic acid in the culture medium. Presently we are trying to obtain deficient mutants in each of the transport systems refered above.

SIXTH SESSION
AMINO ACIDS AND INOSITOL TRANSPORTS

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H. SYCHROVA
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TRANSPORT OF L-GLUTAMATE IN *Schizosaccharomyces pombe*

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In the fission yeast *Schizosaccharomyces pombe* 972h⁻ a measurable L-glutamate transport activity only appears after preincubation of cells with an appropriate energy source, e.g. D-glucose, D-fructose or sucrose. The transport of L-glutamate into the cells harvested from early stationary growth phase and preincubated with 1 % D-glucose (optimum conditions) is practically unidirectional and is mediated by a single uphill transport system with a K_T of 170 μmol/litre and a J_{max} of 4.8 nmol per min per mg dry wt. It was shown to be a rather nonspecific system since all the amino acids tested, including basic, neutral and acidic ones, behaved as potent competitive inhibitors. The only exceptions were L-proline and 2-aminoisobutyric acid which are not transported into the cells under the assay conditions. The system has a pH optimum at 3.0-4.0, the accumulation ratio of L-glutamate is highest at 0.6-1.0 mg dry wt. per ml and decreases with increasing L-glutamate concentration in the external medium. The system present in cells preincubated with D-glucose is unstable and its activity decays after cell washing with water or stopping cytosolic protein synthesis with cycloheximide with a half-time of 24 min. This decay is significantly retarded by phenylmethyl sulfonyl fluoride, a serine proteinase inhibitor. Since the cycloheximide treatment is accompanied only by a decrease of J_{max} of L-glutamate transport, leaving the K_T unchanged, it may be speculated that this phenomenon is due to a reduction of the effective carrier concentration in the plasma membrane by proteolytic attack.

Uptake of lysine in thiosine-resistant mutants of *Schizosaccharomyces pombe*

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The uptake of lysine in *Schizosaccharomyces pombe* is an active and unidirectional process. Lysine is transported by at least two systems which are not constitutive and differ in affinity and substrate specificity.

Mutants defective in the transport of lysine were isolated and characterized. After UV mutagenesis colonies resistant to thiosine, a toxic analogue of lysine, were isolated. The measurement of uptake of different amino acids in these mutants showed that in most of them the uptake of lysine and arginine was reduced while the transport of leucine or glutamic acid was not affected. Detailed kinetic analysis showed that the half-saturation constant of teh high-affinity system increased in mutant strains significantly compared to the wild type, so that the high-affinity transport system for lysine was specifically affected. This was also confirmed by a complementation test in which all the thiosine-resistant strains with higher K_T for lysine belonged to one complementation group. Kinetic and genetic analysis showed that our mutants were identical with canl.1 mutants, indicating that a single high-affinity system for the transport of basic amino acids and their analogues (lysine, arginine, histidine, canavanine and thiosine) exists in *S. pombe*.

myo-INOSITOL TRANSPORT IN *SACCHAROMYCES CEREVISIAE*

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In order to study the relation between glucose sensing and glucose transport Küntzel et al. screened a yeast library for homologs of human glucose transporters by using synthetic probes [1]. One of the cloned genes (HET1) encodes a 67 kD protein containing twelve membrane-spanning domains and showing 28 to 29% homology to the human hepatoma and yeast SNF3 glucose transporter.
Recently Nikawa et al. reported on an identical gene encoding a *myo*-inositol transporter (ITR2) in *Saccharomyces cerevisiae* [2].
We studied *myo*-inositol transport in a wild-type strain (HK3), a het1(itr2) null mutant (HK 52) and a mutant harboring HET1(ITR2) on a multicopy plasmid (GAL-HET/ABYS).
Inositol transport is most likely an active transport as predicted by Nikawa et al. [3]. Plasmamembrane vesicles prepared from the wild-type strain HK 3 demonstrated no *myo*-inositol countertransport.
From our experimental data there seems to be no indication that inositol and glucose are transported by the same carrier. Both transports behave differently in the mutants and under glucose repression and derepression. In uptake experiments (2min. 25°C) *myo*-inositol was transported in HK 3 cells with a V_{max} of 11.6 (pmol/min)/mg wet weight and a K_m of 86 µM , in HK 52 (het1[itr2]-) cells with a V_{max} of 3.2 (pmol/min)/mg and a K_m of 75 µM and in the GAL-HET/ABYS transformant with a V_{max} of 17.3 (pmol/min)/mg and a K_m of 85 µM . Such significant V_{max} changes in inositol transport between these strains were not found in respective glucose uptake experiments.
Under glucose repression (2% glucose) and derepression (2% galactose) conditions *myo*-inositol uptake in HK 3 wild-type strain demonstrated no significant changes in V_{max} and K_m. Glucose transport at the other hand demonstrates the characteristical changes under derepression: an increase in affinity (K_m 5 to 2 mM) and increase in V_{max} (10 to 20 [nmol/min]/mg).

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SEVENTH SESSION
SUGAR TRANSPORTS

C. A. MICHELS
G. F. FUHRMANN
M. D. LOUREIRO-DIAS
M. HÖFER
M. CORTE-REAL
T. GONÇALVES
I. SPENCER-MARTINS
M. A. HERWEIJER

COMPARISON OF TWO HIGH-AFFINITY MALTOSE PERMEASES FROM *SACCHAROMYCES*. Corinne A. Michels, Frank Cotty and Qi Cheng, Department of Biology, Queens College and the Graduate School of the City University of New York, Flushing, NY 11367.

The *MAL1g* allele is a naturally occurring, partially functional varient of *MAL1* proposed to be the result of a chromosomal rearrangement of the right telomere-linked region of chromosome VII. Based on restriction mapping and Southern analysis, the gene encoding maltose permease at *MAL1g* shares little or no sequence homology with the gene encoding maltose permease at *MAL1*, *MAL6* and all of the other *MAL* loci. To distinguish these two alleles, the gene encoding maltose permease at *MAL1* is refered to as *MAL11-1* and that at *MAL1g* is refered to as *MAL11-2*. In this report, we compare the structure and functional activity of these two species of maltose transporter.

The *MAL11-2* gene encodes a deduced protein of 617 amino acid residues exhibiting considerable hydrophobicity. Computer analysis indicates that the MAL11-2 protein shares 57% identity and 74% similarity to the MAL61 protein, the maltose permease encoded by the *MAL61* gene of the *MAL6* locus. Hydropathy analysis shows that the MAL11-2 protein contains 12 transmembrane domains arranged in two groups of 6 domains each which are separated by a hydrophillic region approximately 71 residues in length. Thus, the MAL11-2 maltose permease, like the MAL61 maltose permease, is a member of the human glucose transporter superfamily which also includes the *Saccharomyces* high-affinity glucose transporters encoded by *SNF3*, *HXT1*, and *HXT2* and the high-affinity galactose transporter encoded by *GAL2*.

Kinetic analysis of the maltose permeases encoded by *MAL11-2*, *MAL11-1* and *MAL61* shows that all are proton symporters and all are high-affinity maltose transporters with a Km of approximately 4mM. In contrast, analysis of the substrate specificity clearly distinguishes these maltose permeases. The MAL11-1 permease is capable of transporting only maltose and turanose while the MAL11-2 permease transports these sugars as well as isomaltose, maltotriose, α-methyl glucoside and palatinose. Neither transports melezitose. Control of the fermentation of various α-glucoside sugars will be discussed in view of these results.

INHIBITION OF GLUCOSE TRANSPORT IN *SACCHAROMYCES CEREVISIAE* BY URANYL IONS

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Transport of glucose into *Saccharomyces cerevisiae* cells occurs by two transport processes. The first transport is mathematically characterized by facilitated diffusion and the second by diffusion kinetics (Fuhrmann et al., Experientia.45, 1018 - 1023, 1989: Fuhrmann and Völker, J.Biotechnology in press). Both transport processes are effectively blocked by uranyl-ions.

Facilitated diffusion of glucose demonstrates a mixed type of inhibition by uranyl ions: Vm decreases and Km increases. The Ki-value for Vm is 43.78 x 10^6 molecules uranyl per cell and the Hill slope 1.64. These values are comparable with the data on uranyl inhibition of glucose consumption by Rothstein et al. (J.Cell. and Comp. Physiol. 32, 261 - 274, 1948) with a Ki-value of 27.21 x 10^6 molecules uranyl per cell and a Hill slope of 2.51. By considering uranyl chemistry a predominant dimeric complex of uranyl might act as a molecule similar to β-D-glucopyranose at the binding site of the carrier and by inhibition of the glucose translocation step. Such an interaction is consistent with a Hill slope of about 2.

The effects on glucose diffusion seem to be more complex. At low concentrations of uranyl a significant inhibition occurs, whereas at higher uranyl concentrations a slight increase is observed. This dual behaviour of uranyl could be due to effects on two different diffusion pathways.

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PHYSIOLOGY OF THE *snf3* MUTANT OF *SACCHAROMYCES CEREVISIAE* IN CONTINUOUS CULTURE

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In *Saccharomyces cerevisiae* the SNF3 has been sequenced and characterized; it was proposed that the gene product is the high-affinity transport system for glucose (Bisson *et al*, 1987. J. Bacteriol. **169**:1656-1662). Recently another gene (HXT2) was proposed to code another high-affinity transport system for glucose in *S. cerevisiae* (Kruckeberg and Bisson, 1990).

Continuous culture is a useful tool to study the regulation phenomenon, since when a steady state is achieved, cells can be harvested under controlled physiological conditions; growth rate is the independent variable.

In parallel fermenters we grew the *snf3* strain and the parental strain in the same mineral medium supplemented with 0.4% glucose and suitable aminoacids, under aerobic conditions.

For all values of dilution rate the concentration in the fermenter was higher for the culture of the mutant. This increase in concentration, as compared with the parental strain supports the hypothesis that a high-affinity transporter is missing in the mutant.

The glucose transfer rate calculated as the ratio between the dilution rate and the yield coefficient was identical in both strains.

Cells from steady state cultures were harvested, centrifuged and washed in ice cold water. Initial uptake rates of labelled glucose were measured in the range 0.5 to 100 mM. Preliminary results indicate that the same kinetic parameters were measured in both strains for the dilution rates tested. Half-saturation constants were in the range 2 and 8 mM.

Our results suggest that the cells have the ability to regulate the uptake of glucose even when the gene product of SNF3 is missing. The only apparent physiological consequence of the absence of the gene product was a loss in the ability to utilize glucose at low concentrations.

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Hexose-Transport-Deficient Mutant of *Schizosaccharomyces pombe*: Phenotype and Genetics

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The important goal of membrane transport studies is the isolation of a particular transport protein and its functional reconstitution in artificial phospholipid bilayers. The most difficult problem of this approach is the identification of the particular protein in a mixture of solubilized membrane proteins, especially if it belongs to constitutive transport systems. In another approach this problem is circumvented by identifying the gene which codes for the transport protein, and by its *in vitro* expression. Our strategy in cloning the gene has therefore been to isolate the transport structural gene of *Sc. pombe* by genetic complementation using a genomic DNA library on a replicating plasmid.

Mutation and Selection. *Schizosaccharomyces pombe* 972h⁻ was grown on a synthetic minimal medium (1) with K-gluconate as the sole carbon source. Cells harvested in the mid log phase (0.2% fresh wt/vol.) were incubated for 30 min with 1.4 mM N-methyl-N'-nitro-N-nitrosoguanidine. For mutants enrichment the method of Megnet (2) was modified as follows: the concentration of 2-deoxy-D-glucose (2-DG) was enhanced to 0.5 mg/ml and the incubation time in batch culture (containing gluconate) was extended to 2 days; mutant colonies were grown on agar plates of the same composition. A total of 4972 2-DG resistant colonies were isolated.

Screening of mutants. 2-DG resistant mutants were screened according to their growth on the following substrates:

GROUP	GLUCOSE	FRUCTOSE	GLUCONATE	GLUCONATE+2-DG	COLONIES
WT	++++	++++	+++	-	WT
WANTED	-	-	+++	+++	WANTED
I	-	-	+++	+++	26
II	-	-	+++	+	7
III	-	-	+++	-	2

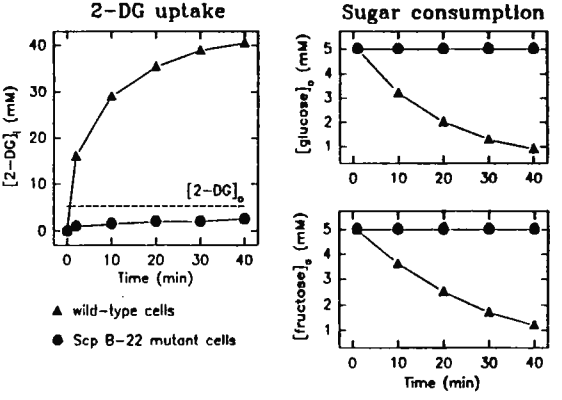
To eliminate the metabolic mutants, the hexokinase activity of crude cell-free extracts was determined. Nine mutants of Group I exhibited hexokinase activity which is comparable to that of the wild type, as shown for *ScpB-22*:

SUBSTRATE	HEXOKINASE (nmol/ml mg/prot.)	
	WILD TYPE	*ScpB-22*
GLUCOSE	322	345
FRUCTOSE	510	523
GLUCONATE	23	20

Phenotype characteristics. The nine mutants meet the criteria of a glucose transport deficiency. Indeed, none of the mutants was capable of taking up glucose and fructose, or of accumulating 2-DG (Fig. 1, data for the mutant *ScpB-22*).

Genetic analysis. The mutant *ScpB-22* was used for a genetic characterization. To eliminate the unwanted (and undefined) mutations, *ScpB-22* was backcrossed at least three times with *Sc. pombe*

Leu⁻h⁺, *Sc. pombe* 975h⁺, and again *Sc. pombe* Leu⁻h⁺, and the method of free spore analysis was applied. The backcrossing experiments indicated that the 2-DG-resistance is not the necessary criterion of the transport deficiency. Consequently, two backcrossed *ScpB-22* strains (their phenotypes in parentheses) were further studied: GDG_6 (Glc⁻Frc⁻2-DG^R Leu⁻h⁻) and GDG_5 (Glc⁻Frc⁻2-DG^S Leu⁻h⁻). The GDG_6 and GDG_5 strains neither took up glucose or fructose, nor accumulated 2-DG; however, there was a slow but measurable uptake of 2-DG in GDG_5.



Tetrade analyses were carried out following the crossing of the two strains GDG_6 and GDG_5 with the wild type *Sc. pombe* 975h⁺. Spores of 23 asci from the GDG_6 crossing, and of 22 asci from the GDG_5 crossing, were separated. After germination of the spores (10 days at 30°C) the phenotype of the tetrades was tested on the following media: synthetic minimal medium (smm) + glucose + leucine, smm + fructose + leucine, smm + gluconate, smm + gluconate + leucine, smm + gluconate + leucine + 2-DG:

SPORES	NUMBER OF SPORES GERMINATED ON SMM + LEU			
	+ GLC + FRC	+ GLC - FRC	- GLC + FRC	- GLC - FRC
PHENOTYPE	WILD TYPE			MUTANT
GDG_6	46	0	0	46
GDG_5	44	0	0	44

Note: THE SEGREGATION OF LEU⁻/LEU⁺ IN THE TETRADE ANALYSIS WAS ALSO 2:2.

However, the segregation of 2-DG resistance of GDG_6 was not 2:2, but rather $2^R:1^S$, confirming the conclusion of the free spore analysis according to which the 2-DG resistance and the transport deficiency are not located at the same gene.

Stable diploid cells for complementation experiments were obtained by crossing the mutant GDG_6 with another one, *mat2-P-lys*, which is deficient in the early meiotic phase. All 109 diploid cells tested exhibited the wild type phenotype. Thus, both the transport defect and the 2-DG resistance are of a recessive character.

Genetic analysis of the two transport-deficient mutant strains derived from *Sc. pombe* 972h⁻, GDG_6, and GDG_5, proved that the mutation was nuclear, monogenic and recessive.

Acknowledgement. This work was supported by the Deutsche Forschungsgemeinschaft, Grant No. Ho 555/12-4.

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TRANSPORT OF GLUCOSE IN A MUTANT OF THE YEAST *Hansenula anomala* WITH POTENTIAL INTEREST FOR THE BIOLOGICAL DEACIDIFICATION OF GRAPE MUST

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As previously reported utilization of L-malic acid by the yeast *Hansenula anomala* was subjected to glucose repression (1). Derepressed mutants with respect to the use of malic acid in conditions similar to those employed in wine making were obtained. These mutant strains displayed inverse diauxy in glucose-malic acid medium (2). In order to elucidate the underlying mechanisms to this diauxic behaviour a study concerned with the transport of glucose in both wild and mutant strains was carried out. We measured glucose transport in the wild strain either in repression or derepression conditions, comparing the activity of glucose transport(s) system(s) of the wild and one of the mutant strains. For the wild strain as derepression conditions we used starvation cells or growth on ethanol, glycerol or malic acid while repression conditions are considered to be mid exponential growth phase on 2% of glucose.

While glucose repressed cells of the wild strain formed a saturable transport system that mediated uptake of glucose, probably by a facilitated diffusion mechanism, in derepressed growth conditions, a second transport system for glucose was induced which appeared to be a proton glucose symport. With respect to the mutant strain, evidence was available that the proton glucose symport activity was present even under glucose repression conditions. As a consequence, during growth of the derepressed mutant strain in a glucose-malic acid medium, two proton symports, one for malate and another for glucose were simultaneously present, their substrates displaying mutual non-competetive inhibition. Furthermore it was observed a significant inhibitor effect of malic acid in the transport of glucose during the first growth phase. These results may contribute to the explanation of the non utilization of glucose in the presence of malic acid and as a consequence of the observed inverse diauxy.

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PITFALLS IN THE MEASUREMENT OF GLUCOSE TRANSPORT IN *SACCHAROMYCES CEREVISIAE* ...

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The purpose of our work was to compare the transport of glucose in ethanol and glucose-grown cells of *S. cerevisiae*. The initial uptake rate (10s) of the sugar was measured in several strains, in a concentration range of 0.1 to 25 mM glucose, at 26°C. It was impossible to use higher glucose concentrations (up to 250 mM) because high blank values (higher than 10% of the sample) were obtained and so the results had to be disregarded.

When the cells were grown on 2% ethanol up to an O.D. of 0.8 (exponential phase) and then harvested and washed with distilled water, we found two transport systems, one with a Km of 3-5 mM and the other with Km>10 mM. When these cells were incubated during 2h with 4% glucose the low affinity system disapeared. Likewise, when the cells were grown on 2% glucose, only one transport system (the high affinity one) was detected.

In ethanol grown cells it was observed that the low affinity transport system was only present when the cells, after being harvested, were kept on ice for one hour. Experiments with 2-deoxy-glucose showed that the high affinity transport system was completely inhibited while the low affinity transport system was not affected by this inhibitor. In glucose grown cells it was not possible to follow the appearance of the low-affinity transport system when the cells were kept on ice for the referred period. But, after this incubation on ice there was a little increase in the Km.

MODES OF LACTOSE UPTAKE IN *KLUYVEROMYCES MARXIANUS*

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During a survey conducted on twelve lactose-assimilating strains of *Kluyveromyces marxianus* and related species *K. lactis* three types of behaviour were found[1], in lactose-grown cells, with respect to lactose uptake mechanisms: I (eight strains)-lactose transported by an apparent proton symport mechanism, transport modes for glucose and galactose variable. It corresponds to described lactose transport systems in this microorganism; II (two strains)- no apparent lactose-proton symport, glucose and galactose transported by an apparent proton symport, and III (two strains)- neither sugar was apparently transported by a proton symport. From the last group one strain, *K. marxianus (bulgaricus)* IGC 2948, was selected for further studies.

The lactose uptake system in *K. marxianus* IGC 2948 was inducible and subject to regulatory effects by glucose (catabolite repression and activation). Transport of labelled lactose by lactose-grown cells, harvested in mid-exponential phase of growth, displayed Michaelis-Menten kinetics with the following parameters at 25°C, pH 5: V_{max}= 0.7-0.8 mmoles/h.g dry weight, K_s= 0.6-0.7 mM). Initial lactose uptake did not affect membrane polarization as measured with a fluorescent dye. Galactose inhibited the uptake of labelled lactose. The same results were observed with other β-galactosidase inhibitors. Simultaneous addition of lactose and glucose or galactose caused extracellular alkalinization of the yeast suspension in water.

Lactose uptake in this strain was shown to proceed mainly via two different coexisting, but essentially alternative mechanisms: lactose is hydrolysed on the cell surface, by an exocellular β-galactosidase, and the resulting products of hydrolysis enter the cell through a passive transport system; and lactose is transported by a proton symporter directly into the cell and is hydrolysed intracellularly.

[1] Carvalho-Silva, M., Spencer-Martins, I. (1990), Antonie van Leeuwenkoek **57**: 77-81

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Do baker's yeast strains have different sugar transport characteristics as compared with laboratory strains?

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The aim of this study was to characterize sugar transport in one of our baker's yeast strains. Since baker's yeast is optimized for growth with high yield on molasses, we were interested to compare glucose and fructose transport systems of our strains with literature data on sugar uptake systems in laboratory strains.

Most authors report that in Saccharomyces cerevisiae, fructose and glucose are transported by the same carriers. The sugars are taken up by facilitated diffusion. However, one brewer's yeast strain has been reported to have a specific H^+/fructose symport [Cason et al., 1986].

Using a sensitive pH-detection assay, developed to study maltose permease kinetics, we could not detect a H^+/fructose symport in our baker's yeast strain. Further studies were performed with ^{14}C-glucose and ^{14}C-fructose.

Contrary to what has been reported by most authors, the results of our measurements could be adequately described by a one-K_m uptake system. We could not fit the data with a low and a high affinity glucose carrier. For glucose transport we measured a K_m of 5-8 mM, and for fructose transport a K_m of 10-20 mM.

To compare *in vitro* measurements of transport with uptake kinetics *in vivo*, yeast was grown in continuous culture at different growth rates. When the kinetic constants obtained from *in vitro* experiments were used to calculate the glucose uptake in the fermentor, an underestimation of the uptake rate was found. This discrepancy can be explained in several ways.

1. we wrongly interpreted our *in vitro* data as containing no high-affinity uptake system.
2. *in vitro* measurements with radioactive substrates do not adequately predict the *in vivo* sugar uptake rates.

Arguments will be presented that the second explanation is the most likely.

Cason, D.T., Spencer-Martins, I., Van Uden, N. (1986) FEMS Microb Lett 36, 307-309

EIGHTH SESSION
MODELS AND REGULATION

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THE CHANGE OF ACTIVITIES OF SOME VACUOLAR TRANSPORT SYSTEMS DURING THE YEAST GROWTH

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The yeast *Candida lipolytica* oversynthesizes citric acid the excretion mechanism of which is unknown. At the same time, the vacuoles of the yeast *S. pastorianus* have the citrate transport system [1] whose physiological significance is not quite clear. The question was whether the *C. lipolytica* vacuoles have the citrate transport system and if it is involved in citrate excretion, presumably, by way of exocytosis. To elucidate this, we isolated vacuoles from the *C. lipolytica* cells which enhance the production of citric acid severalfold (Table 1). ATPase of the *C. lipolytica* vacuoles features high activity which goes down during the growth. Simultaneously, the values of ATP-dependent ΔpH and membrane potential (E_m) generated at the tonoplast were observed to decrease. It is significant, however, that the values of ΔpH and E_m were comparable with those sufficient to provide citrate and α-ketoglutarate transport in the *S. pastorianus* vacuoles. What is more, transport of Ca^{2+} which was several times as intensive as that of citrate did not significantly decrease in the *C. lipolytica* vacuoles whereas the citrate transport disappeared completely. The latter was observed only at the beginning of *C. lipolytica* growth. Interestingly, the vacuoles of the yeast *C. lipolytica* grown under carbon deficit when the citrate secretion is insignificant also showed the decrease in the ATPase activity, in the values of ΔpH

MISUSE OF NONLINEAR EADIE-HOFSTEE PLOTS

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Eadie-Hofstee plots are often used for the analysis of kinetic parameters from biological systems which follow a saturation kinetic, mathematically described by Michaelis-Menten (Eqn.1), as enzyme reactions.
Analysis of such systems in which two saturable processes are involved leads to nonlinear Eadie-Hofstee plots. It has become the practice to analyse the two quasilinear slopes, assuming each slope being described by a form of Eqn.2.
Resolution of nonlinear plots cannot be done by eye, as has been shown for nonlinear Scatchard plots (1). Parameters determined by graphical analysis of nonlinear Eadie-Hofstee plots are often in erroneous.
Eadie and Hofstee transformed the Michaelis-Menten equation (Eqn.1) to a linear form which easily allows the extraction of the unknown parameters graphically by plotting V versus V/S (Eqn.2).

$$v = \frac{V_M * S}{K_M + S} \qquad (1)$$

$$V = -K_M * \frac{V}{S} + V_M \qquad (2)$$

If two Michaelis-Menten kinetics are involved, the system can be described by the following equation:

$$v = \frac{V_{M1} * S}{K_{M1} + S} + \frac{V_{M2} * S}{K_{M2} + S} \qquad (3)$$

$$V = -K_{M1} * \frac{V}{S} + V_{M1} + V_{M2} * \frac{K_{M1} + S}{K_{M2} + S} \qquad (4)$$

From conversion to the Eadie-Hofstee form (Eqn.4) it is obvious that no simple addition of linear functions is obtained and therefore no linearisation of quasilinear slopes is possible. The same is shown for addition of one Michaelis Menten term and a simple diffusion (Eqns.5 and 6).

$$V = \frac{V_M * S}{K_M + S} + S * K_D \qquad (5)$$

$$V = -K_M * \frac{V}{S} + V_M + K_D * (K_M + S) \qquad (6)$$

In *Saccharomyces cerevisiae*, several transport systems were characterized by analysis of such nonlinear plots.
In these cells glucose transport, as shown by countertransport experiments, occurs by facilitated diffusion (net flux = influx - efflux). It is necessary to keep the uptake period short enough (for example 5 sec.) in order not to have a significant efflux. Only under these conditions initial uptake experiments can be evaluated by the equations given above.
Linearization of two quasilinear slopes in Eadie-Hofstee plots of initial uptake experiments in *Saccharomyces cerevisiae* has led to the prediction of two glucose transport systems, one is constitutive with a K_M value of about 20 mM ("low affinity system") and one with a K_M value of 1-2 mM ("high affinity system") (2).
In Fig.1 kinetics of two facilitated diffusion systems with K_{M1} = 4 mM and V_{M1} = 40 (nmol/min)/mg (dashed line), K_{M2} = 20 mM and V_{M2} = 100 (nmol/min)/mg (dotted line), and addition of these processes (solid line) are generated. Fig. 1a shows the S vs. V plot, Fig. 1b the Eadie-Hofstee plot. The values are chosen to simulate a "high affinity" and a "low affinity" glucose uptake system as proposed by Bisson (2) and others. It is obvious that, in the range of normally used substrate concentrations, graphical determination of parameters by linearization of the two slopes does not represent the generated curve.

Fig.2 demonstrates the behaviour of one facilitated diffusion system with K_M = 4 mM and V_M = 40 (nmol/min)/mg (dashed line), a simple diffusion with K_D = 0.1 (µl/min)/mg (dotted line), and addition of these systems (solid line). The so called "low affinity system" of Bisson and others appeared in our analysis by computer assisted nonlinear regression (3) to be a first order term which we interprete as a simple diffusion.
Graphical analysis of nonlinear Eadie-Hofstee plots is often complicated by low V_M-values. Then, the slope designated as "high affinity" slope becomes rather short (Fig.3) and in combination with normal scattering of the data points it is difficult to detect this slope. This might have led to the interpretation that the "high affinity" glucose transport is absent in some *snf3* mutants of *Saccharomyces cerevisiae* (4).
Graphical analysis of kinetic parameters has been developed before computer assisted nonlinear regression was possible. This regression analysis is the best way to obtain more precise kinetic parameters. However, if graphical methods are used, the extracted parameters should be taken to regenerate a new curve, which should be in agreement with the original one.

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Fig. 1a

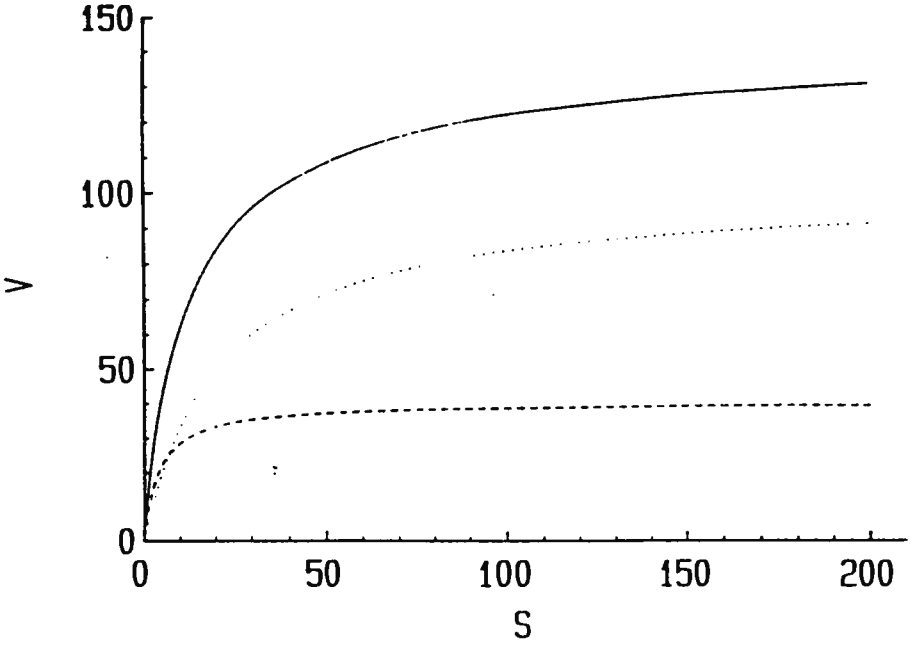


Fig. 1b

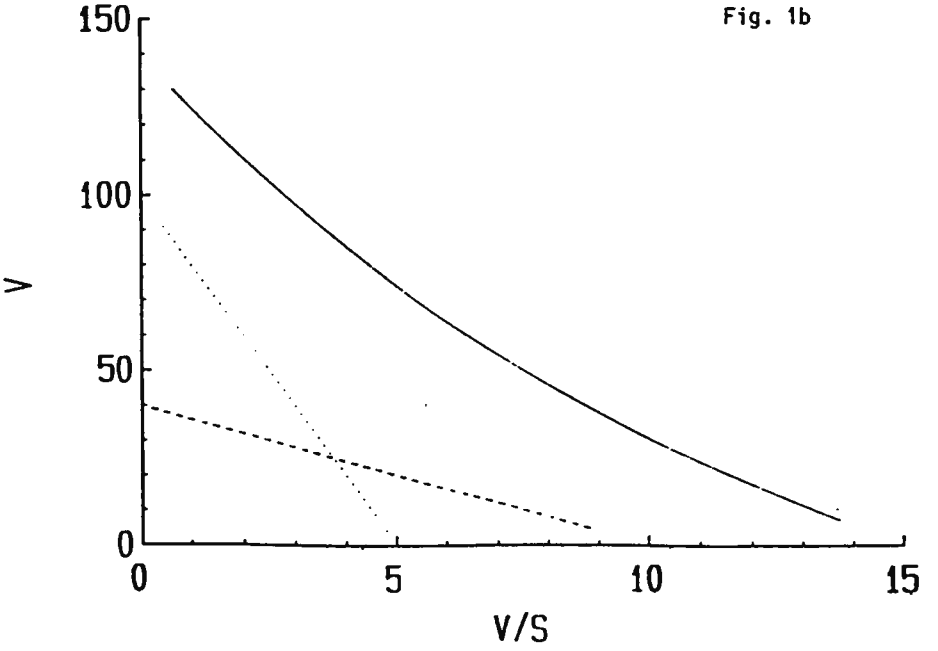


Fig. 2a

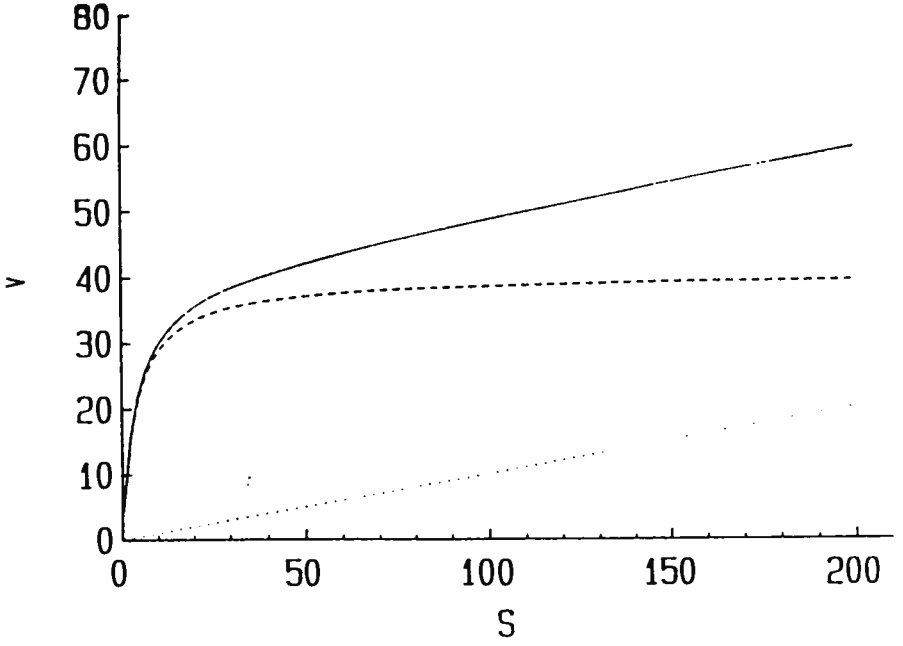


Fig. 2b

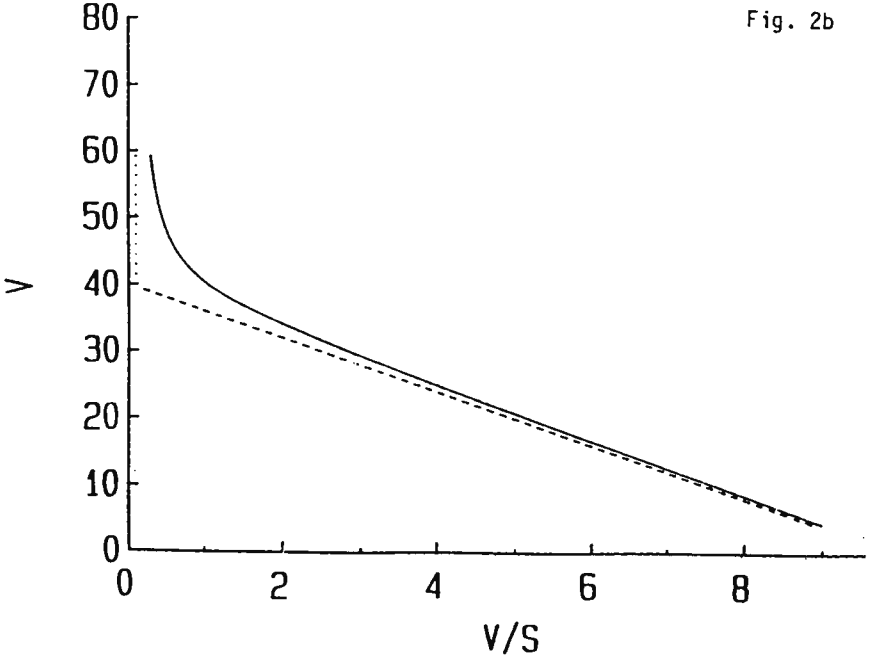
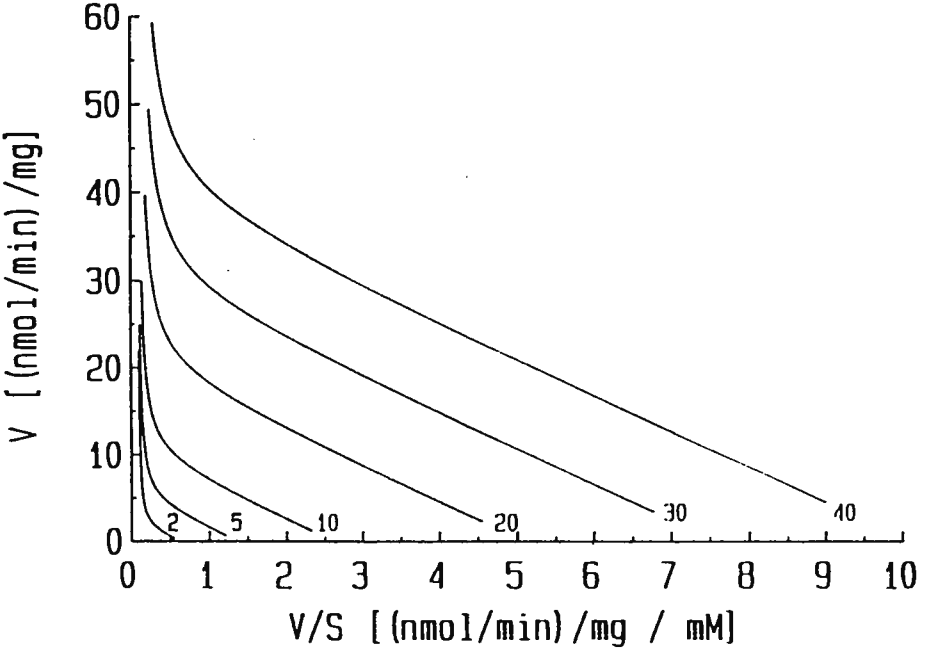


Fig. 3



OPTIMIZATION OF MEMBRANE TRANSPORT MODELS USING EVOLUTIONARY STRATEGIES

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The degree of natural optimization of special qualities of biological systems during evolution can be investigated by an application of mathematical optimizing techniques to appropriate models (Honig & Stein, 1978; Burbaum *et al.*, 1989). We tested a simple algorithm, based on the theory of evolutionary strategies (Rechenberg, 1973), on two different transport-models. The optimizing process utilizes principles of biological evolution, e.g. mutation, selection, and recombination. This method has already been successfully applied to the construction of medical devices, to problems in economy, and to the investigation of neural networks.

In principle, the algorithm works as follows: First, a quality Q which should be optimized is defined as a function of special parameters. In each step (generation) of the algorithm the initial parameter values are altered randomly by small amounts (mutation), while the original parameter values are retained in memory. Second, the values of Q belonging to the altered and the unaltered set of parameters are compared. The set of parameters which gives an improved value of Q is then selected as the new initial point of the next generation (selection). The whole procedure is devided into phases with different sizes of mutation steps, and is terminated after a certain number of generations.

A model of a symporter is shown in Fig. 1. The signs ' and " denote the two sides of a membrane, A and B are the substrates, Ki are the dissociation constants and kj are the rate constants. The unidirectional flux of A, divided by the total concentration of symporter molecules (the quality Q), was maximized as a function of dissociation constants K1 and K2, as well as of all rate constants kj, with A'=B'=1 and A"=B"=0. All parameter values were initially set to 1, and during optimization restricted to the interval (1,10). The dissociation constants K3 and K4 were calculated using two Wegscheider relations of the system. The quality Q reached maximum for dissociation constants approaching 1 (the lower limit) and rate constants in both directions approaching 10 (the upper limit).

On the other hand, the accumulation ratio A'/A" at a steady state (Q) was maximized as a function of dissociation constants K1 and K2 and of all rate constants, with B'=10 and B"=1. In this case the quality Q reached maximum for dissociation constants K2 and K4 and rate constants k3 and k4 approaching 1, and rate constants k1 and k2 approaching 10. Rate constants k3 and k4 represent a leakage flux of A which reduces the accumulation ratio. After several reiterations of the optimizing process the standard deviations of the remaining parameters remained high. Obviously, these parameters do not affect the optimization of the quality Q. It can indeed be shown analytically that they do not determine the value of Q. Therefore, no asymmetry with regard to the dissociation constants is required to give a high accumulation ratio.

Under the given conditions, the unidirectional flux of A and the accumulation ratio A"/A' cannot be maximized at the same time, since the values of k3 and k4 have to be large in the first case and small in the second case.

A model of a carrier is shown in Fig. 2. Here again, A denotes the substrate, and L" is a ligand restricted to side ". If the concentration of L" is oscillating or fluctuating around a mean value, the system is able to do work, i.e. a net flux of A from ' to " exists, even if A'=A". The precondition of this behavior is that the fluctuations be external, i.e. that they are imposed on the system (this could also be shown by Monte Carlo simulations), and that there exists an asymmetry with regard to the rate constants (Westerhoff *et al.*, 1986; Chen 1987). Starting with A'=A"=1 M we maximized the increase in concentration of A" (Q) after a given time interval as a function of rate constants (Fig. 3). Their values were again restricted to the interval (1,10) and k6 was calculated using the Wegscheider relation. The concentration of L" was determined by a Mäander-function, oscillating between 0 M and 1 M with a frequency of 1/s. The calculation yielded these optimal values for the rate constants: k1=4.0, k2=1.5, k3=2.0, k4=5.0, k5=10.0, k6=4.3, k7=4.0, k8=10.0. With these values given, in equilibrium the concentrations of C' and CAL" are almost

equal and low, and those of CA' and CL" are almost equal but high. It is surprising, that these values do not represent the highest possible asymmetry. What we here observe is perhaps a phenomenon of resonance.

The aims of a future work will be a further development of the optimizing algorithm, and its application to more complex transport models (including those which combine several transport systems). In this way, we hope to come to a better understanding of the designing principles of the membrane transport systems.

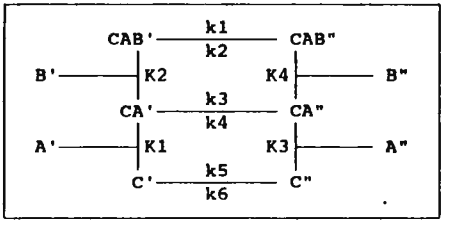

Fig. 1: (s. text)

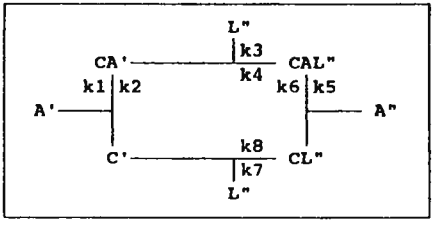

Fig. 2: (s. text)

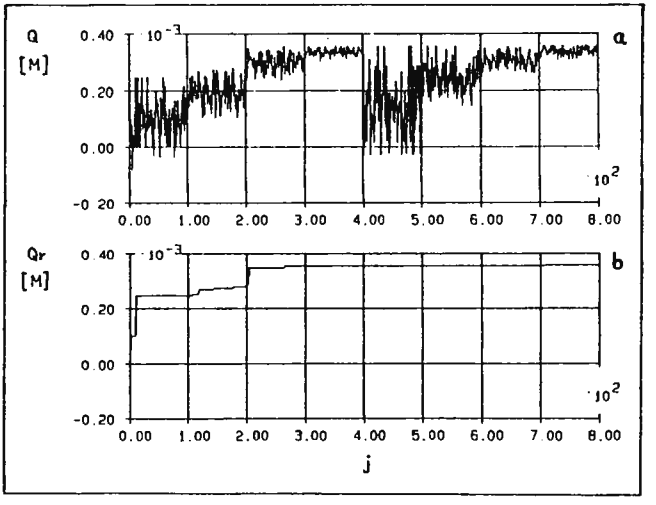

Fig. 3: Increase in concentration of A" in M (the quality Q in a) and the actual best value of Q (Qr in b) as functions of the generation (j). The mutation step-size was changed every 100 generations. The values were: 4, 2, 1, 0.5, 4, 2, 1, 0.5.

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KINETIC ANALYSIS OF THE PLASMA MEMBRANE H^+ATPase OF YEAST: DEPENDENCE ON EXTRACELLULAR pH

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In a first part of a kinetic analysis of the plasma membrane H^+ATPase of yeast it was shown (1) that the model with the reaction sequence:

$$- E2 - E1 - H^+E1 - H^+E1\cdot ATP - H^+E1\text{-}P\cdot ADP - H^+E1\text{-}P - H^+E2\cdot Pi - E2\cdot Pi - E2 -$$

describes the pH dependence of K_m(ATP) (2) and, with K(E1/E2)<1, the high Km(ATP) at low K_d(ATP) (3). On the basis of K(E1/E2) shift a kinetic hypothesis of the so called "glucose activation" (4) was developed. Intra- and extracellular intrinsic (thermodynamic) pKs of 5.5-6 and 2.5-3 respectively were derived in agreement with the Neurospora enzyme (5). The second part of the kinetic analysis deals with the dependence of the H^+ATPase in the glucose activated cellular state on the extracellular pH. Calculation yields an apparent (kinetic) extracellular pK of 1.5-1.95, which does not agree with experimental results. Half maximal initial extracellular acidification rates ($V_{max}H^+/2$) are measured at pH 3.9-4.2 (absence of K^+) which agrees with pKa 3.9 in proteoliposomes co-reconstituted with purified S.c. H^+ATPase and bacteriorhodopsin (6). Saturating K^+concentrations shift $V_{max}H^+/2$ down to pH 3.3-3.8 and accelerate acidification rates, suggesting that the membrane potential affects both the extracellular pK and the turnover of the ATPase. Calculations, using also an expanded reaction sequence with separated steps of H^+translocation and E-P hydrolysis, show that besides the extracellular pK either also the intracellular pK or one of the E1-E2 transition must be dependent on the membrane potential.

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THE GLUCOSE INDUCED RAS-MEDIATED cAMP SIGNALING PATHWAY IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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The addition of fermentable sugar to derepressed yeast cells causes a rapid signal-like spike in the cAMP level which, based on studies with temperature-sensitive mutants in cAMP synthesis, appeared to trigger a protein phosphorylation cascade. Evidence was obtained that induction of the cAMP signal by fermentable sugar is mediated by the CDC25-RAS-adenylate cyclase pathway. In this way, fermentable sugar was identified as the first physiological activator of the RAS-adenylate cyclase pathway in yeast. Detection of fermentable sugar in the medium is obviously of crucial importance to yeast cells. Activation of the RAS pathway is only triggered by fermentable sugars and only in concentrations ($K_{1/2}$ = ±15-20 mM) which result in fermentation of sugar to ethanol. Lower concentrations of these sugars are metabolized by means of respiration and do not activate the pathway. Therefore, the RAS pathway in yeast appears to serve as a specific sensor for the detection of fermentable sugars in concentrations appropriate for fermentation. Whether the low-affinity glucose carriers play an active role in the sensing mechanism or whether there exists a special receptor for the glucose is unclear. Whatever the nature of the glucose receptor, it clearly has a much lower affinity for glucose than the sugar kinases or the high-affinity sugar transport system. A deletion mutant in the *SNF3* gene, which encodes a component of the high-affinity glucose transport system, is not affected in glucose-induced cAMP signaling. Recently, two mutants (*fdp1* and *byp1*) specifically affected in glucose-induced cAMP signaling and one mutant (*lcr1*) affected both in glucose- and acidification-induced cAMP signaling (but not in basal cAMP synthesis) have been identified. Recent results on the cloning of the *FDP1*, *BYP1* and *LCR1* genes and the function of their gene products will be presented. Evidence has also been provided that the transient nature of the cAMP increase is due to feedback-inhibition by cAMP-dependent protein kinase.

Intracellular acidification also stimulates the RAS-adenylate cyclase pathway. This occurs at a point downstream of the first part of the pathway which is only required for stimulation by fermentable sugar. Hence, intracellular acidification apparently constitutes another physiological trigger of the RAS-adenylate cyclase pathway in yeast. This makes sense in view of the stimulating effect of the pathway on the mobilization of reserve carbohydrates and the generation of energy. Increased energy production is an obvious requirement to overcome stress conditions and under such conditions intracellular acidification might generally be expected to occur in yeast cells. Acidification-induced stimulation of the RAS-adenylate cyclase pathway might constitute a rescue mechanism for cells suffering from stress conditions. Recent results concerning the connection between the glucose-induced RAS-cAMP pathway and the pathway responsible for activation of plasma membrane H^+-ATPase will be discussed.

The discovery of fermentable sugar as trigger for the RAS pathway in yeast appeared to support the generally held, but poorly substantiated idea that nutrients act as stimulators of this pathway. Recent studies however with glucose repression mutants and with mutants affected in derepression showed that the sugar-induced activation pathway of RAS-adenylate cyclase is a glucose-repressible pathway. The glucose-induced cAMP signal is absent in glucose-repressed wild type cells and in cells of mutants (*cat1*, *cat3*) unable to show derepression. In addition, mutants lacking glucose repression such as *hxk2* and *hex2* and mutants with strongly reduced activity of cAMP-dependent protein kinase showing deficient glucose repression, show glucose-induced stimulation of cAMP synthesis when grown on glucose. All results obtained in this respect are consistent with the presence of a glucose-repressible protein in the pathway, although this protein has not yet been identified. The finding that the glucose-activation pathway of RAS-adenylate cyclase is glucose-repressible has important consequences. It confines the physiological role of this pathway to the transition period between the respirative-gluconeogenic growth mode and the fermentative growth mode. A second consequence is that the pathway cannot act as a trigger for progression over the 'start' point of the yeast cell cycle since the pathway is no longer operative during exponential growth on glucose. Although the glucose-induced RAS-cAMP pathway does not appear to be responsible for the main glucose repression mechanism, the pathway has a common initiation point with the pathway responsible for glucose repression.

CONCLUSIONS AND PERSPECTIVES

Alonso Rodríguez-Navarro

CONCLUSIONS AND PERSPECTIVES

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Energetics and transport through membranes have been extensively studied in all types of living cells during the past three decades. Still, the incorporation of the recombinant-DNA technology as a normal tool in biological research has produced a significant boost in the field.

This workshop has shown, once more, that almost every technique used in the research of energetics and membrane transport can be used with yeast. This variety of the experimental methods together with the extensive information on its biology and the simplicity of its genetic manipulation make yeast an invaluable tool for the research on membrane functions. Furthermore, the information obtained with yeast can be used as a basic models for other cell-walled eucariotic cells, as those of plants, in which basic research presents more difficulties.

An important disadvantage in the use of yeast cells in transport studies is the actual difficulty of using them for electrophysiology. The small size of yeast cells make it difficult to impale them with electrodes, and basic data, as the membrane potential, are still unknown for yeast researchers. A significant breakthrough in the study of electrical processes in the yeast membrane is the patch clamp technique. The two communications on patch clamp in the workshop show that the technique is ready for extensive studies. However, patch clamp may not be sufficient to investigate many electrical processes of the plasma membrane of yeast. Researchers in the field must pursue alternative techniques.

A consequence of the unanimous consensus on the utility of the workshop was the unanimous decision of holding a new meeting next year, the tenth of the series. Since science progresses posing new questions, the tenth will not be the last. And we hope that in a near future we will have the opportunity to meet again in a new Workshop organized by Fundación Juan March.

LIST OF INVITED SPEAKERS

Workshop on
YEAST TRANSPORT AND ENERGETICS

List of Invited Speakers

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Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop on The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on The Polymerase Chain Reaction.**
Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

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