an anaerobic growth
calc calculated
crit critical
exp experimental
tr transient

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Kinetic analysis and simulation of glucose transport in plasma membrane vesicles of glucose-repressed and derepressed *Saccharomyces cerevisiae* cells

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Summary. In this study experimental data on the kinetic parameters investigated by other authors[1–5,11] together with own data on plasma membrane vesicles, have been subjected to a computer simulation based on the equations describing facilitated diffusion. The simulation led to an ideal fit describing the above data. From this it can be concluded that glucose is transported by facilitated diffusion, and not by active transport as was postulated by Van Steveninck[14,15].

The simulation method also demonstrates that the fast sampling technique used by these authors[1–5,11] underestimates the fluxes. Thus, the parameters given do not contribute to the understanding of glucose transport under different metabolic conditions.

The K value of plasma membrane vesicles prepared from glucose-repressed cells is around 7 mM. Derepression, particularly by galactose, causes a highly significant increase in affinity as shown by a decrease in the K value to 2 mM. The highest affinity was measured in a triple kinaseless mutant grown on glycerol with a K value of 1 mM. If seems, therefore, that the kinetic parameters derived from initial uptake rates of glucose in intact cells[1–5,11] using single flux analysis, such as Eadie-Hofstee- or Lineweaver-Burk-plots, are in error.

Key words. Saccharomyces cerevisiae; growth conditions; kinaseless mutant; plasma membrane vesicles; glucose transport; kinetics and computer simulation.

Introduction

Kinetic analysis of glucose transport in *Saccharomyces cerevisiae* cells grown under different metabolic condition has so far been reported for intact cells only[1–5,11]. From these studies it has been postulated that glucose can be transported both by a transporter with a high affinity and a low apparent K_m (1–3 mM) and one of low affinity and high apparent K_m (20–100 mM). The low affinity process occurs, according to these investigators, by carrier-mediated facilitated diffusion. Examples for this process are transport of glucose under nonmetabolizing conditions (i.e., in iodo-acetate-inhibited cells) or in triple kinaseless mutants. For the high affinity process, the involvement of the internal enzymes hexokinase (PI and PII) and glucokinase has been discussed; alternatively, a vectorial phosphorylation process such as an active transport analogous to the phosphotransferase system has been proposed[14,15]. Kinase-positive cells containing one or more of the hexokinase isoenzymes (PI and PII) and glucokinase exhibited biphasic transport kinetics, with a high affinity (1–3 mM) and a low affinity (20–

100 mM) transporter[1-4,11]. The conclusion has also been drawn that the high affinity glucose transport in intact cells is under general glucose repression control[1]. In this investigation we analyzed the kinetics of glucose transport by making use of the phenomenon of countertransport in isolated plasma membrane vesicles under ice bath conditions. This method allows a complete elimination of metabolic processes, in contrast to the above-cited experiments using intact cells, where the consumption of glucose cannot be exactly analyzed and therefore the inside concentration cannot be determined. Our kinetic data for the vesicles have been analyzed in two different ways by conventional differential equations for facilitated diffusion as given by Wilbrandt[16]. The first method uses the measured inside and outside concentrations of glucose at the maximum of countertransport for the estimation of the half-saturation concentration, and the second analysis was accomplished by computer-assisted simulation of the transport processes under question.

The plasma membrane vesicles were derived from *Saccharomyces cerevisiae* cells grown in batch cultures. Cells were harvested in the middle and at the end of the exponential growth phase with glucose and in the middle of the exponential growth phase with galactose or glycerol as substrate. Cells grown on sugars were the strain H1022 and those grown on glycerol a triple kinaseless mutant D-308.3 tk–. Our results are in agreement with the assumption of the predominance of a facilitated diffusion transport mechanism in vesicles as well as in intact cells. We have been able to describe mathematically not only the experimental results in plasma membrane vesicles but also the published data of the authors cited above. Our computer fitting analysis does not confirm the conclusions drawn by these investigators.

Materials and methods

Strains and growth conditions. The strains of *Saccharomyces cerevisiae* used in this study were H1022, ETH Zürich, and the triple kinaseless mutant D-308.3 tk–. The cells were grown in batch cultures under aeration at 30 °C. The composition of the growth medium was 2% peptone, 1% yeast extract and 2% carbohydrate.

Vesicle preparation. Plasma membrane vesicles were prepared as described by Kreutzfeldt and Fuhrmann[10].

Transport experiments. Transport experiments with 2% plasma membrane vesicles were carried out under ice bath conditions in 0.4 M KCl solution adjusted to pH 4.5. As shown by Fuhrmann et al.[9] plasma membrane vesicles are only sealed at pH 4–5; at pH 7, for example, the vesicles are leaky. Diffusion of glucose at the appropiate pH values is small compared to transport, and negligible in countertransport experiments. The amount of glucose in the vesicles was analyzed by ^{14}C labeled glucose. Separation of the vesicles was done by the Millipore filter technique as described previously[9,10].

Analysis of transport kinetics. Transport and countertransport of glucose was analyzed by conventional rate equations[17].

$$d_s/dt = V_m S_o/(S_o + R_o + K_s) - V_m S_i/(S_i + R_i + K_s) \quad (1)$$

and

$$d_r/dt = V_m R_o/(R_o + S_o + K_r) - V_m R_i/(R_i + S_i + K_r) \quad (2)$$

where s and r are the amounts of cold and ^{14}C labeled glucose and S_i, R_i and S_o, R_o are the concentrations of unlabeled and labeled sugars inside (i) and outside (o) the vesicles. Countertransport can be demonstrated when S_i, R_i and S_o, R_o belong to the same molecular species[16,17]; R_i and R_o are labeled and in tracer concentrations, and S_i (S_o) is unlabeled and in a relatively high concentration (iso-countertransport). Thus, the half-saturation constants are $K_s = K_r = K$. A further advantage is the high osmotic pressure of the 0.4 M KCl solution in relation to the initial concentration gradient of about 47 mM unlabeled glucose. By assuming ideal osmotic behavior of the vesicles the volume change resulting from glucose transport is only 6%, so that amounts and concentrations of glucose are closely related.

For calculation and computer-assisted simulation we used equations for the concentration changes inside and outside the vesicles. Therefore, the following mathematical transformation was applied[7,8]:

$$ds_i/dt = ds_i/dS_i \times dS_i/dt \quad (3)$$

For ideal osmotic behavior the following correlation exists[7,8]:

$$ds_i/dS_i = n(S_o + N)/(S_o + N - S_i)^2 \quad (4)$$

were n is the osmotic content of the vesicle population and N the osmotic concentration of the electrolyte solution. The right side of the equation represents the volume of the vesicle population at time t. Thus equation 1) becomes:

$$dS_i/dt = (S_o + N - S_i)^2/n(S_o + N) \quad (5)$$
$$((V_m S_o/(S_o + R_o + K) - V_m S_i/(S_i + R_i + K))$$

because R_i and R_o are osmotically not relevant tracer concentrations. The term $(S_o + N - S_i)^2/n(S_o + N)$ is the reciprocal of the vesicle volume ($1/V_i$) and can be used to determine the fraction of the total vesicle volume in relation to the outside volume of the suspension medium.

Computer-assisted simulation of Michaelis-Menten kinetics. We used a computer simulation program written by K.-H. Röhm[13], which applies the 4th-order Runge-Kutta-Merson formula[12]. The program solves simultaneously the following 4 equations:

$$dS_o/dt = 1/V_o(V_m S_i/(S_i + R_i + K) - V_m S_o/(S_o + R_o + K))$$

$$dS_i/dt = 1/V_i(V_m S_o/(S_o + R_o + K) - V_m S_i/(S_i + R_i + K))$$

$$dR_o/dt = 1V_o(V_m R_i/(R_i + S_i + K) - V_m R_o/(R_o + S_o + - K))$$

$$dR_i/dt = 1/V_i(V_m R_o/(R_o + S_o + K) - V_m R_i/(R_i + S_i + K))$$

Figure 1 shows a model experiment for flux simulation with these 4 equations. The glucose tracer concentrations R_o and R_i, which are initially in equilibrium, are transiently changed by a coupling mechanism at the carrier by the gradient of unlabeled glucose represented by S_o and S_i. By the time the gradient *has vanished* both processes, the countertransport of R_i into the vesicles and the efflux of S_i from the vesicles, have disappeared. Because of the large volume of the flux medium (about 98% in relation to the 2% vesicle volume) the concentration changes of the tracer in R_o are barely detectable. For demonstration purposes the steep change of the gradient S_i to S_o into equilibrium is only shown for the last minutes.

Materials. Peptone from casein and yeast extract were purchased from E. Merck, Darmstadt, FRG. D-(U-^{14}C)glucose, sp. act. 4 mCi/mmol, was obtained from Amersham Buchler, Braunschweig, FRG. All other chemicals were of analytical grade quality.

Results

Relation between growth conditions and countertransport maximum. The bar graph in figure 2 demonstrates differences in the countertransport maximum t_{max} between plasma membrane vesicles prepared from cells grown in batch cultures on glucose to the middle of the exponential growth phase (first bar), at the end of the first exponential growth phase (second bar), and from cells grown to the middle of the exponential growth phase on galactose (third bar). The differences are highly significant, with p between the first two columns and the second and the third column being smaller than 0.001. The cells from which the vesicles have been prepared differ in metabolic state; the first group of cells were glucose repressed, whereas cells of group 2 changed to glucose derepression, and group 3 were grown in the presence of galactose, which efficiently prevents respiratory repression by its *inefficiency* in acting on the control system for cAMP formation[6]. The differences in countertransport maximum are directly related to the affinity of the carrier, as shown by Wilbrandt[16].

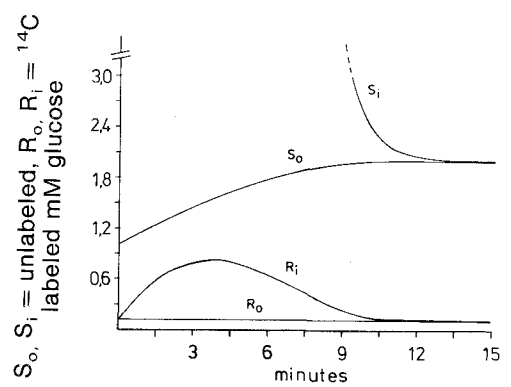

Figure 1. Changes in glucose concentration with time of S_o, S_i, R_o and R_i according to equations dS_o/dt, dS_i/dt, dR_o/dt and dR_i/dt. Vesicle volume 2%, K value = 2 mM, V_m = 0.24 mmol glucose/ I vesicles/min. Initial concentrations: S_o = 1 mM, R_o and R_i = 0.125 mM.

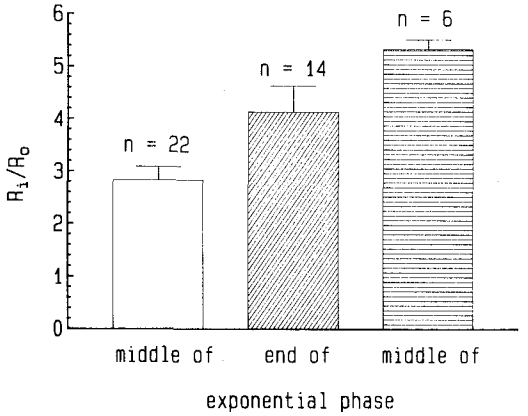

Figure 2. Bar graph of experimentally determined t_{max} values of countertransport in plasma membrane vesicles. The vesicles were prepared from cells grown on glucose to the middle and to the end of the exponential growth phase, and from cells grown on galactose to the middle of the exponential phase.

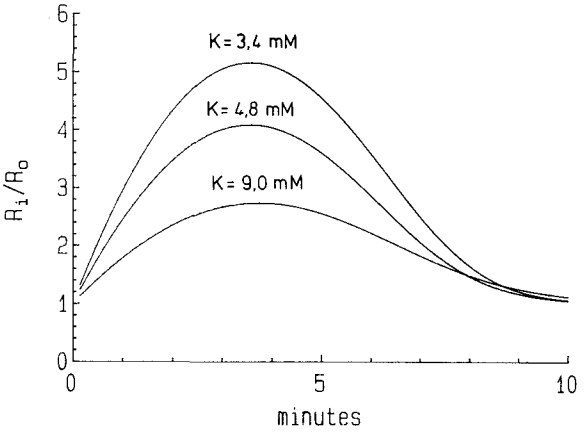

Figure 3. Simulation of the countertransport experiments in figure 2. Simulation as in figure 1 except that the K values are 3.4, 4.8 and 9.0 mM according to t_{max} values in figure 2. The concentrations of R_o and R_i were plotted as ratio R_i/R_o.

Figure 3 simulates the countertransport experiments from above. The conditions for countertransport were similar to those given in figure 1. According to the t_{max} values of the first bar in figure 2, the affinity of the transport system in the vesicles is low, with a K value of about 9 mM. The K values in the second and third col-

umn can be simulated by K values of 4.8 and 3.4 mM.

Determination of K values by flux measurements. According to Wilbrandt[17] the K values in countertransport can be evaluated at the t_{max} of countertransport by:

$$K = (S_i - (R_i/R_o)S_o)/((R_i/R_o) - 1) \quad (6)$$

This relation is easily obtained by considering that at t_{max} of countertransport the rate of dR_i/dt becomes zero. Therefore, in addition to the countertransport experiment, we measured in a separate experiment movement of glucose S_i down its gradient to determine the inside concentration of S_i at t_{max}. S_o was also calculated, from the dilution at the beginning and the equilibrium distribution at the end of the experiment. The table gives the result from vesicles which were prepared from cells grown glucose-repressed and galactose-derepressed as in figure 2, and thirdly from vesicles prepared from the kinaseless triple mutant grown in glycerol.

Determination of K values by simulation of countertransport and efflux data. Figures 4, 5 and 6 show the measuring points for the countertransport and efflux in the experiments listed in the table. In contrast to the profile of simulated countertransport, as depicted in figures 1 and 3, the countertransport experiments in vesicles demonstrate an asymmetrical broad left-shifted peak value. An explanation for this distribution can be given by the fact that the vesicle population is not uniform in size. In freeze-fracture electron microscopic images of vesicles, different sizes are seen[9]. In order to account for the different sizes we simulated the experimental countertransport curve by mixing populations with different V_m values in constant proportions. Small vesicles should demonstrate higher transport rates because of the greater surface in relation to volume, and the opposite would be true for larger vesicles. As can be seen from figures 4, 5 and 6, the countertransport curves can be correctly fitted by a curve. As can be theoretically expected, the K values for a mixed population should be lower than those predicted from the t_{max} value. In figure 4, by mixing 13 populations with V_m values between 1.92 and 0.03 we obtained a K value of 7. In figure 5, 13 populations with the same range as in figure 4 were mixed at a K value of 2. In figure 6, only 7 populations ranging from V_m 0.72 to 0.09 were mixed at a K value of 1.

In contrast to the good simulation of the experimental values for countertransport, the efflux values deviate significantly in their initial phase. The deviation is more pronounced in experiments with high affinity. There are methodical problems with the Millipore filter technique which could account for this deviation; despite fast separation of vesicles from the suspension medium, the filter-

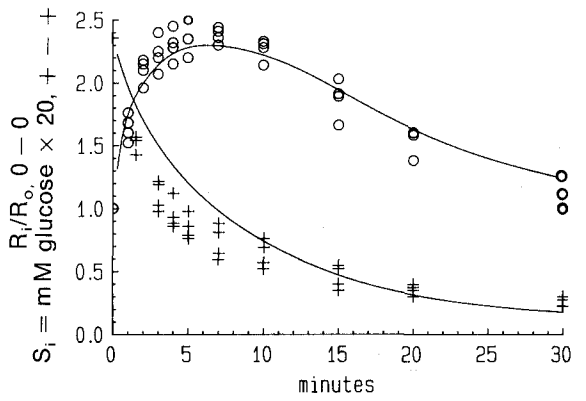

Figure 4. Efflux and countertransport in plasma membrane vesicles prepared from cells grown in glucose to the middle of the exponential phase. Single experimental values from 4 experiments. The curve through the points was fitted by simulation as described in the text.

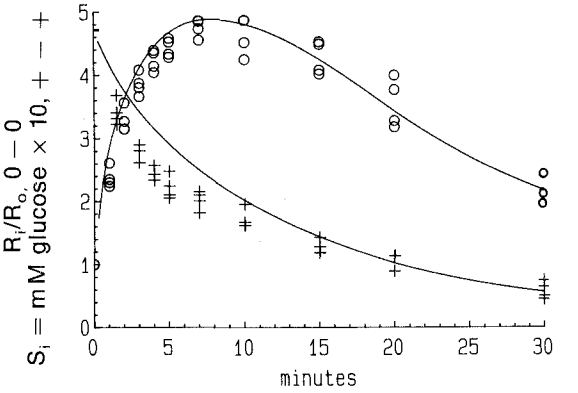

Figure 5. Efflux and countertransport in plasma membrane vesicles prepared from cells grown in galactose to the middle of the exponential phase. For other details see figure 4.

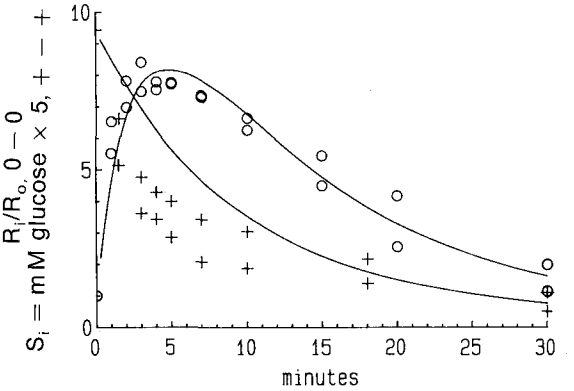

Figure 6. Efflux and countertransport in plasma membrane vesicles prepared from a triple kinaseless mutant grown in glycerol to the middle of the exponential growth phase. Single experimental values of two experiments. For other details see figure 4.

K values calculated at t_{max} (Wilbrandt[17])

Plasma membrane vesicles prepared from yeast cells	mean ± SD (4) K (mM)	Simulation K (mM)
Glucose repressed	8.6 ± 1.5	7
Galactose derepressed	2.9 ± 0.6	2
Kinaseless mutant	1.8 ± 0.9	1

$K = S_i - (R_i/R_o)S_o/(R_i/R_o) - 1$

ing and washing on the filter takes about 30 s, and this time is not short enough to prevent a significant drop in the initially high glucose concentration inside the vesicles, due to an efficient transport system. In the second half of the efflux the initial gradient is mainly exhausted and the separation time of the Millipore filter technique is sufficient.

Simulation of glucose transport in intact cells. Our simulation program was used to fit experimental data of fast uptake experiments of sugars in intact cells as used by the cited authors[1-5,11]. For example, we mimicked an experiment of Bisson and Fraenkel[2] by using fast sampling periods, every 5 s from 5 to 20 s in order to obtain, as the authors claim, true initial rates. From their figure 3A we chose the data for the wild type DFY1 and of the triple kinaseless mutant DFY 437. The wild type shows, as in many other examples, biphasic behavior in Eadie-Hofstee-plots as reviewed in the introduction. For simulation we used osmotic conditions and the percentage of the cells as given. The range of glucose concentrations was 1–200 mM and a K value of 1.5 mM and V_m of 15 nmol min^{-1} mg^{-1} (wet wt) was used as predicted from the so-called high affinity slope in this experiment. For simulation in figure 7, it was assumed that inside glucose concentration is kept near zero by efficient glucose metabolism. The simulation clearly shows that under these circumstances also, a biphasic Eadie-Hofstee-plot is obtained. Thus, the linearization in the Eadie-Hofstee-plot is prone to artifacts, especially at high glucose concentrations.

On the other hand, the curve obtained for the triple kinaseless mutant in figure 3A by Bisson and Fraenkel[2] was fitted by the assumption that glucose metabolism is ineffective, so that glucose concentration rises inside according to the facilitated diffusion equation. By using for simulation the same K value of 1.5 mM and a V_m of 15, as above, the shape of the experimental curve from the triple mutant in intact cells is adequately reproduced by

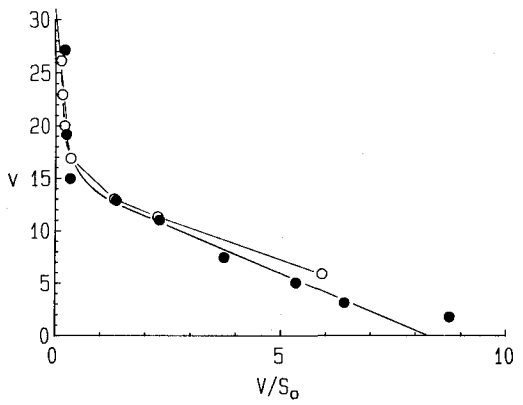

Figure 7. Simulation of glucose transport in intact cells with effective glucose consumption by metabolism. Experimental data taken from Bisson and Fraenkel[2], figure 3A for the wild type DFY1. Closed circles represent the experimental data, open circles were generated by simulation as described in the text.

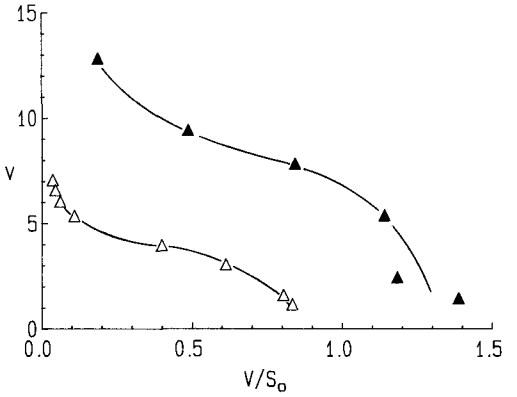

Figure 8. Simulation of glucose transport in intact cells without glucose consumption by metabolism. Experimental data taken from Bisson and Fraenkel[2], figure 3A for the triple kinaseless mutant DFY 437. Closed triangles represent the experimental data, open triangles were generated as described in the text.

our simulation method (fig. 8.) The differences in the abscissa of figures 7 and 8 should be noted; the curves in figure 8 are steeper than in figure 7. By ignoring the nonlinear slope using a linear regression analysis, the slope representing the K_m value in the Eadie-Hofstee-plot would be 9.2 mM for the experimental data of Bisson and Fraenkel and 6.2 mM according to the simulation. The two values are not significantly different because of nonlinearity, but they are significantly different from the K_m value of 1.5 mM, which was used for simulation. Thus, the evaluation of kinetic parameters by the fast sampling procedure in intact cells as used by the cited authors[1-5,11] and by the Eadie-Hofstee-plot is erroneous.

Discussion

The kinetic analysis of glucose transport in intact *Saccharomyces cerevisiae* cells as well as in plasma membrane vesicles prepared from these cells is in agreement with the kinetics of facilitated diffusion. This is clearly demonstrated in figures 3–8. Thus an active transport, as suggested by Van Steveninck[14,15], can be excluded. There is no indication in nature that at the same time both active transport and a passive facilitated diffusion process are operative for the same substrate species.

The kinetic analysis of glucose transport in intact cells, as reported by the cited authors[1-5,11] as well as by others who used the fast sampling technique and similar methods are in error because they analyze experimental data for a single flux kinetic, for example by the Eadie-Hofstee- or Lineweaver-Burk-plots. As shown by the simulation method, and as predicted earlier[6] when an efficient facilitated diffusion mechanism is operative, as analyzed in these investigations and in those cited, it is impossible to measure true initial influxes only. The inside concentration of the sugar, S_i, already rises in less than a second to significant values, because of the effi-

cient transport and the small size of the cellular compartment. Thus, in the case of metabolic depletion in kinaseless mutants or in experiments on the transport of non-metabolizable sugar, the kinetic parameters derived by single flux analysis are erroneous. A possibility of analyzing such data is, however, offered by our simulation technique.

Only if glucose metabolism is much faster than transport, so that the glucose inside concentration is kept near zero, does fast sampling technique in intact cells together with single flux evaluation give a reasonable estimation of the kinetic parameters. However, this is only true when low glucose concentrations are added to the outside medium. As can be seen from figure 7, at high glucose outside concentrations (over 10 mM), a second slope can artificially be produced. This slope has been considered by the authors cited [1-5, 11] as the low affinity flux in the biphasic behavior of their kinetic analysis. The understanding of the basic principle of glucose transport, especially under different metabolic conditions, has been greatly hampered by these erroneous interpretations.

Our results demonstrate kinetic data for glucose transport in plasma membrane vesicles which were prepared from cells grown under different metabolic conditions. For the first time a clear-cut dependence of glucose transport on metabolism is shown. According to our analysis, glucose-repressed cells grown aerobically have a K value of 7 mM. There is a significant decrease of the K value towards a higher affinity if the cells are harvested towards the end of the first exponential growth phase. A further increase in affinity is obtained when cells are grown in galactose. Plasma membrane vesicles prepared from cells grown in galactose to the middle of the exponential phase show a comparably higher affinity of 2 mM for the K value.

The highest affinity, however, was measured in plasma membrane vesicles which were prepared from a triple kinaseless mutant grown on glycerol up to the middle of the exponential growth phase. The K value was estimated to be around 1 mM. This result is clearly in contrast to the opinion of Bisson and Fraenkel and other investigators [1-5, 11], who assumed, as a result of underestimation of facilitated transport kinetics, K_m values between 20 and 100 mM.

Because the plasma vesicles have different sizes, an estimation of V_m is rendered more difficult. The experimental curves, however, suggest a tendency for plasma membrane vesicles prepared from the triple mutant to show higher V_m values. This could suggest that not only the affinity of the transport system, but also the carrier concentration, might increase under such metabolically deprived conditions.

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