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Low- and High-Affinity Transport Systems for Citric Acid in the Yeast *Candida utilis*†

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Citric acid-grown cells of the yeast *Candida utilis* induced two transport systems for citric acid, presumably a proton symport and a facilitated diffusion system for the charged and the undissociated forms of the acid, respectively. Both systems could be observed simultaneously when the transport was measured at 25°C with labelled citric acid at pH 3.5 with the following kinetic parameters: for the low-affinity system, V_{max}, 1.14 nmol of undissociated citric acid s^{-1} mg (dry weight) of cells^{-1}, and K_m, 0.59 mM undissociated acid; for the high-affinity system, V_{max}, 0.38 nmol of citrate s^{-1} mg (dry weight) of cells^{-1}, and K_m, 0.056 mM citrate. At high pH values (above 5.0), the low-affinity system was absent or not measurable. The two transport systems exhibited different substrate specificities. Isocitric acid was a competitive inhibitor of citric acid for the high-affinity system, suggesting that these tricarboxylic acids used the same transport system, while aconitic, tricarballylic, trimesic, and hemimellitic acids were not competitive inhibitors. With respect to the low-affinity system, isocitric acid, L-lactic acid, and L-malic acid were competitive inhibitors, suggesting that all of these mono-, di-, and tricarboxylic acids used the same low-affinity transport system. The two transport systems were repressed by glucose, and as a consequence diauxic growth was observed. Both systems were inducible, and not only citric acid but also lactic acid and malic acid may induce those transport systems. The induction of both systems was not dependent on the relative concentration of the anionic form(s) and of undissociated citric acid in the culture medium. Undissociated citric acid entered the cells slowly by simple diffusion in glucose-grown cells. The permeability of the cells to undissociated acid by simple diffusion increased with pH, with the diffusion constant increasing 100-fold between pHs 3.0 and 5.0.

Of the approximately 500 yeast species which are currently recognized, about one-half have strains that are able to use citric acid as a carbon and energy source (1). *Candida utilis* is one of these yeast species. This implies that in such strains, citric acid is able to cross the plasma membrane, as either the anion(s) or the undissociated acid or in both forms. Surprisingly, no articles about plasma membrane transport of citric acid in yeasts seem to have been published. Besides its academic interest, the subject may have a practical dimension, since citric acid, given its nontoxic nature, has long been used as an acidulent in the food industry (5). Furthermore, yeasts may cause food spoilage by consuming organic acids, and *Candida* species have been shown to be food-contaminating flora (5).

In this article, we report on the transport of citric acid across the plasma membrane in a strain of the yeast *C. utilis* and provide experimental evidence indicating that citric acid-grown cells of this yeast induce two mediated transport systems for citric acid, presumably a proton-citrate symport and a facilitated diffusion system for the undissociated form of the acid.

MATERIALS AND METHODS

Microorganism and growth conditions. *C. utilis* IGC 3092 was originally received from the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, The Netherlands, under the designation CBS 890. According to Lodder and Kreger-van Rij (9), the strain had been received from S. Windisch under the name *Candida arborea* and is probably a fodder yeast. It was maintained on a medium containing glucose (2%, wt/vol), peptone (1%, wt/vol), yeast extract (0.5%, wt/vol), and agar (2%, wt/vol). *C. utilis* was grown in a mechanically agitated fermentor (Minifermenter-System; Biolab, B. Braun Melsungen AG) with pH and temperature control. For growth, a mineral medium with vitamins (10) and a 0.5% (wt/vol) concentration of a carbon source (glucose, citric acid, lactic acid, malic acid, ethanol, or glycerol), at the pH values given in Results, was used. All cultivations were carried out at 25°C.

Measurement of initial uptake rates. Cells were harvested in the mid-exponential phase, centrifuged, washed twice with ice-cold distilled water, and suspended to a final concentration of about 45 mg (dry weight) per ml.

For estimating uptake rates of labelled citric acid, 10 μl of the yeast suspension was mixed in conical tubes with 30 μl of 0.1 M KH_2PO_4 buffer at the desired pH value. After 2 min of incubation at 25°C in a water bath, the reaction was started by the addition of 10 μl of an aqueous solution of [1,5-^{14}C]citric acid (about 2,100 dpm/nmol) at the desired concentration and stopped by dilution with 5 ml of ice-cold water. The sampling times for citric acid-grown cells were 0, 5, and 10 s. The sampling times for glucose-grown cells were 0, 30, and 60 s. The reaction mixtures were filtered immediately through GF/C membranes (Whatman, Inc., Clifton, N.J.), washed on the filters with 5 ml of ice-cold water, and counted in a scintillation fluid (OptiPhase HiSafe II; LKB [FSA Laboratory Supplies, Loughborough, England]). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer, with correction for disintegrations per minute.

Initial uptake rates were also estimated by measuring proton uptake with a standard pH meter (PHM 82; Radiometer A/S, Copenhagen, Denmark) connected to a flatbed Perkin-Elmer 024 recorder (Perkin-Elmer Corp., Norwalk,

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Conn.). The pH electrode was immersed in a water-jacketed chamber with a 10-ml capacity kept at 25°C and provided with magnetic stirring. To the chamber, 4.5 ml of 10 mM KH_2PO_4 and 0.5 ml of the yeast suspension were added. The pH was adjusted to the desired value, and a baseline measurement was obtained. The desired amount of carboxylic acid (adjusted to the experimental pH value) was added, and the subsequent alkalization was monitored with the recorder. The slope of the initial part of the pH trace was used to calculate the initial uptake rate. Calibration was performed with HCl.

Measurement of citric acid accumulation. Citric acid-grown cells (20 μl; 45 mg [dry weight] per ml) were added to 60 μl of 0.1 M KH_2PO_4 at desired pH and also to 60 μl of buffer containing carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The reaction was started by the addition of 20 μl of 19.6 mM [1,5-^{14}C]citric acid (about 2,100 dpm/nmol). At appropriate times, 10 μl was taken from the reaction mixture and filtered immediately through Whatman GF/C membranes. The filters were washed twice with 5 ml of ice-cold water, and radioactivity was counted as indicated above. The intracellular concentration of citric acid was calculated under the assumption that 1 mg (dry weight) of the yeast contained 2.0 μl of intracellular water (6).

Estimation of glucose and citric acid concentrations. Glucose concentrations were estimated by the glucose oxidase method (Test-Combination; Boehringer GmbH, Mannheim, Germany). Citric acid concentrations were estimated by the UV method (Test-Combination).

Calculation of concentrations. Concentrations of the several ionization forms of carboxylic acids were calculated by the Henderson-Hasselbach equation with the following pK values: citric acid, $pK_1 = 3.13$, $pK_2 = 4.76$, and $pK_3 = 6.40$; isocitric acid, $pK_1 = 3.29$, $pK_2 = 4.71$, and $pK_3 = 6.40$; malic acid, $pK_1 = 3.40$ and $pK_2 = 5.05$; fumaric acid, $pK_1 = 3.02$ and $pK_2 = 4.38$; lactic acid, $pK_1 = 3.86$; acetic acid, $pK_1 = 4.76$; and pyruvic acid, $pK_1 = 3.79$.

Calculation of kinetic parameters for the mediated transport systems. Estimates of kinetic parameters were obtained from the Lineweaver-Burk plots of the initial uptake rates of radiolabelled citric acid as well as the plots of the initial rates of proton disappearance in the external buffer. The K_m for total citric acid was based on the concentrations of both anionic and undissociated citric acid. The K_m values for facilitated diffusion of the undissociated acid or for the proton-citrate symport were based on the concentration of undissociated citric acid or anionic forms, respectively, that would be present at the experimental pH value.

Reproducibility of the results. All of the experiments were performed at least three times, and the data reported here are the average values.

Chemicals. Radioactively labelled [1,5-^{14}C]citric acid was obtained from the Radiochemical Centre (Amersham, Buckinghamshire, United Kingdom) and had a specific activity of 110 mCi/nmol. All other chemicals were reagent grade and were obtained from commercial sources.

RESULTS AND DISCUSSION

Proton-citrate symport in citric acid-grown cells. Transient external alkalization indicative of proton uptake was observed when citric acid was added to a suspension of cells in weak buffer (pH 5.0) that had been grown on citric acid (0.5% [wt/vol] at pH 4.8). Lineweaver-Burk plots of the initial rates of proton disappearance in the external buffer, calculated from the initial slopes of the pH curves, as well as plots of the initial rates of uptake of radiolabelled citric acid were linear over the entire experimental concentration range (Fig. 1). This indicated that the uptake mechanism obeyed Michaelis-Menten kinetics and that the transport of citric acid was mediated by a saturable carrier, probably a proton symport. From the plot for labelled substrate uptake, the following kinetic parameters were calculated as described in Materials and Methods: V_{max} (at pH 5.0), 0.58 nmol of total citric acid s^{-1} mg (dry weight)$^{-1}$; and K_m (at pH 5.0), 0.112 ± 0.007 mM total citric acid. Similar results were obtained when the transport was measured at higher pH values (up to 6.0). Using the hypothesis that an H^+-citrate symport was present, we found a K_m of 0.111 ± 0.007 mM at pH 5.0 and a K_m of 0.310 ± 0.005 mM at pH 6.0 for citrate. If facilitated diffusion was present, K_m would be $0.54 \times 10^{-3} \pm 0.03 \times 10^{-3}$ mM and $0.17 \times 10^{-4} \pm 0.003 \times 10^{-4}$ mM for undissociated citric acid at pH 5.0 and 6.0, respectively. The data suggest that the transport of citric acid at such pH values occurred via a proton-citrate symport. To substantiate this hypothesis, accumulation studies were performed. Transport of labelled citric acid at pH 5.0 was accumulative (Fig. 2). After about 5 min, 90% of the accumulated radioactive substrate had not been metabolized, since the addition of

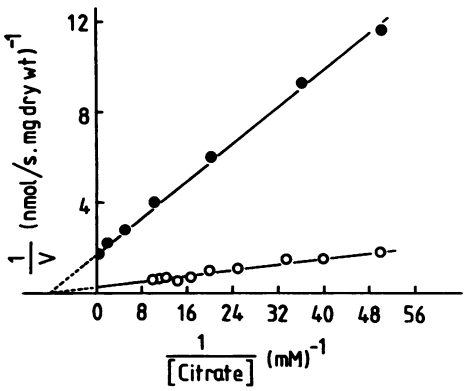

FIG. 1. Lineweaver-Burk plots of initial rates of uptake of labelled citric acid and protons by citric acid-grown cells of *C. utilis* IGC 3092 at pH 5.0 as a function of citrate concentration. Symbols: ●, labelled citric acid; ○, protons.

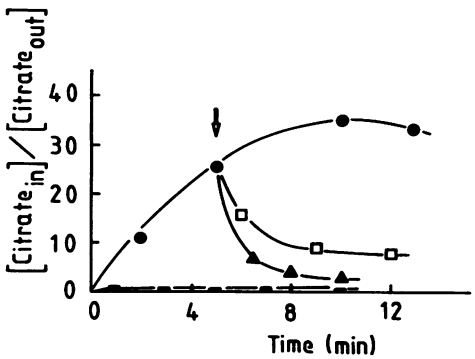

FIG. 2. Accumulation of labelled citric acid (●) by citric acid-grown cells of *C. utilis* IGC 3092 at pH 5.0. The initial extracellular concentration of total citric acid was 3.9 mM. At the time indicated by the arrow, samples of the suspension received the following: ▲, cold citric acid to a final concentration of 0.2 M; or □, CCCP to a final concentration of 0.5 mM. ■, CCCP added to the reaction mixture before the addition of labelled citric acid.

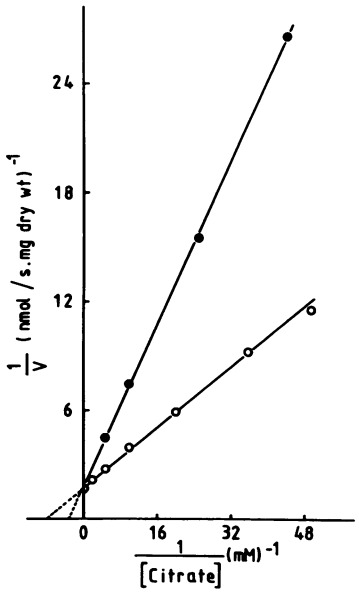

FIG. 3. Lineweaver-Burk plots of initial uptake rates of labelled citric acid at pH 5.0 as a function of citrate concentration. Symbols: ○, absence of other tricarboxylic acid; ●, in the presence of 6 mM isocitric acid.

cold citric acid induced counterflow to this extent (Fig. 2). The accumulation ratio in terms of nonmetabolized citric acid was about 40. The protonophore CCCP prevented accumulation and induced rapid efflux of accumulated nonmetabolized citric acid (Fig. 2). The observed accumulation, while consistent with the hypothesis of the existence of a proton symport, does not constitute final proof, since simple or facilitated diffusion of the undissociated acid would display similar accumulation. However, as reported earlier for *Candida sphaerica* (4), the rapid efflux of accumulated radioactive citric acid that was observed after the addition of CCCP indicates that transport and accumulation of labelled citric acid were dependent on the transmembrane proton motive force and that the tricarboxylic acid carrier observed at pH 5.0 is a proton-tricarboxylate symporter that allows uphill transport and accumulation as a function of pH. At present we are trying to elucidate the proton-citrate stoichiometry and answer the question of whether only one, two, or all types of citrate ions are transported by the symporter.

Isocitric acid was a competitive inhibitor of citric acid transport at pH 5.0, suggesting that this acid was transported by the same carrier as citric acid (Fig. 3). Indeed, this acid induced proton uptake that followed Michaelis-Menten kinetics as a function of the concentration of the acid. Aconitic, tricarballylic, hemimellitic, and trimesic acids did not act as competitive inhibitors of citric acid at pH 5.0, nor did they induce proton uptake, indicating that these tricarboxylic acids were not transported at pH 5.0 by citric acid-grown cells. Pyruvic acid, acetic acid, fumaric acid, and L-malic acid inhibited the uptake of labelled citric acid in a noncompetitive way while displaying proton uptake when used as substrates (not shown). This suggests that these carboxylic acids probably used proton symports other than the proton-citrate symport and inhibited the latter by competing for protons.

Simultaneous presence of low- and high-affinity citric acid transport systems in citric acid-grown cells. The initial transport rate of citric acid, also assayed in washed cells that had been grown on citric acid medium (0.5% [wt/vol] at pH 4.8) was measured at pH values lower than 5.0 (from pH 4.0 to pH 2.0) with radioactive citric acid at concentrations up to 3.9 mM. The Lineweaver-Burk or Eadie-Hofstee plots of the initial velocities were biphasic, with a low-affinity component. Figure 4 shows the results of a typical experiment at pH 3.5. This biphasic plot behavior is consistent with the presence of two distinct permeases, and since the relative concentration of undissociated acid (which is nearly absent at pH 5.0) increases with decreasing extracellular pH, it is conceivable that in this lower pH range the H^+-citrate symport described above coexisted with an operating low-affinity transport system for the undissociated acid. Transport of labelled citric acid at pH 3.5 was also accumulative, with the accumulation ratio in terms of nonmetabolized substrate being about 100 (results not shown). To distinguish between the two possible transport modes for each permease, it is thus necessary to ascertain the form of the acid that is transported. Estimates of the Michaelis constants could be calculated for each permease at several pH values (Table 1). The variation of the K_m values between pH 2.0 and 5.5 for the high-affinity component (up to 0.5 mM) was over 3,000-fold when K_m was calculated as the concentration of undissociated acid and less than 15-fold when it was expressed as the concentration of citrate ions, suggesting that these charged forms of the acid were transported. With respect to the low-affinity component (0.5 to 3.9 mM), the

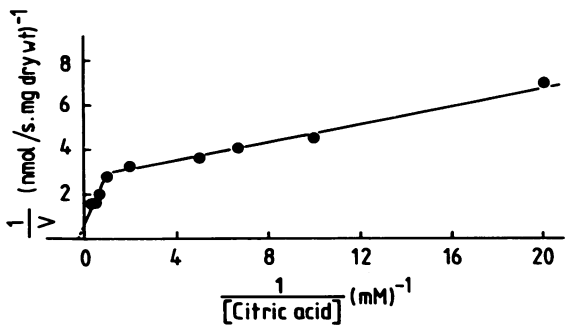

FIG. 4. Lineweaver-Burk plots of initial rates of uptake of labelled citric acid at pH 3.5 by citric acid-grown cells of *C. utilis* IGC 3092 as a function of citric acid concentration.

TABLE 1. Michaelis constants (K_ms) for citric acid transport systems as a function of pH in *C. utilis* IGC 3092

pH_{out}[a]	K_m (mM) for:			
	High-affinity system		Low-affinity system	
	Anions[b]	Undissociated acid[c]	Anions (mM)	Undissociated acid
2.0	0.034	0.458	0.151	2.035
3.0	0.025	0.033	0.697	0.815
3.5	0.056	0.023	1.454	0.588
4.0	0.021	0.0024	1.283	0.147
5.0	0.111	0.00053	NM[d]	NM
5.5	0.253	0.00015	NM	NM
6.0	0.310	0.00002	NM	NM

[a] pH_{out}, extracellular pH.
[b] Transport of anionic form(s).
[c] Facilitated diffusion of undissociated form.
[d] NM, not measurable.

TABLE 2. Inhibition of initial uptake rates of labelled citric acid by *C. utilis* IGC 3092 after incubation for 30 s with 100 μM CCCP at different pH values

pH_{out}[a]	Inhibition (%) of initial uptake rates of labelled citric acid at concn of:	
	0.1 mM	3.9 mM
1.5	Nearly absent	Nearly absent
2.0	63	38
3.0	79	52
5.0	82	75

[a] pH_{out}, extracellular pH.

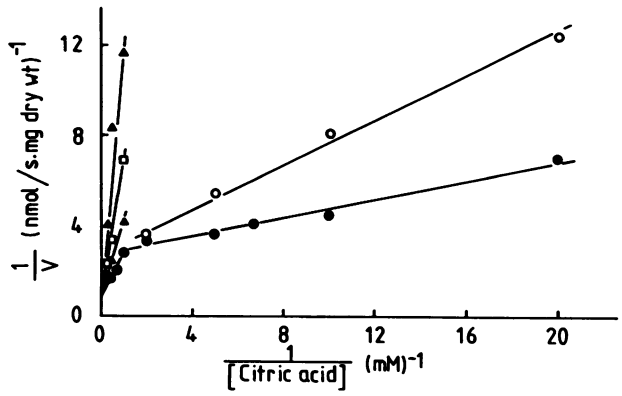

FIG. 5. Lineweaver-Burk plots of initial uptake rates of labelled citric acid at pH 3.5 as a function of citric acid concentration. Symbols: ●, absence of other carboxylic acid; ○, presence of 6 mM isocitric acid; ▲, presence of 60 mM isocitric acid; □, presence of 100 mM L-lactic acid; △, presence of 60 mM L-malic acid.

variations of the K_m values between pHs 2.0 and 4.0 were similar for the two hypotheses and not inconsistent with facilitated diffusion of the undissociated acid. To substantiate the hypothesis of facilitated diffusion, estimates of initial uptake rates of labelled citric acid were obtained after incubation for 30 s with 100 μM CCCP at pH values lower than 5.0 (Table 2). The inhibition induced by the addition of CCCP was much less pronounced in the higher than in the lower experimental citric acid concentration, being nearly absent at pH 1.5 with both citric acid concentrations tested. At this pH value (relative concentration of the citrate, <2.3%), the Lineweaver-Burk plot of the initial velocities was linear over the entire experimental concentration range, and although the transport of labelled citric acid was accumulative, the addition of CCCP did not induce a rapid efflux of accumulated nonmetabolized citric acid (data not shown). The results led us to conclude that the citric acid transport corresponding to the low-affinity component (Fig. 4) was not dependent on the transmembrane proton motive force and that therefore the acid was transported in the undissociated form by facilitated diffusion. The initial transport rates were also measured by monitoring H^+ uptake by a cell suspension after citric acid addition at pHs lower than 5.0, as described in Materials and Methods. The Lineweaver-Burk plot of the initial rates of proton disappearance in the external medium, at pH 4.0, was linear over the experimental range of 0.02 to 0.3 mM (data not shown). At higher citric acid concentrations, it was not possible to estimate proton uptake rates, probably because of the buffering capacity of the citric acid itself. Furthermore, at pH values lower than 4.0, given the high extracellular proton concentrations, it was not possible to test the presence of H^+ uptake associated with citric acid uptake.

The question of whether the high- and low-affinity citric acid transport systems exhibited distinct substrate specificities arose. At pH 3.5, isocitric acid was a competitive inhibitor for the high-affinity system (Fig. 5), and L-malic acid and L-lactic acid acted as noncompetitive inhibitors for the same system (data not shown). At this pH value, as was observed at pH 5.0, aconitic, tricarballylic, hemimellitic, and trimesic acids did not show any inhibitory effect. This suggested that isocitric acid shared the high-affinity citric acid system, which appeared to be in all likelihood the proton-citrate symport described above. With respect to the low-affinity transport system, not only isocitric acid but also L-lactic acid and L-malic acid competitively inhibited citric acid at pH 3.5 (Fig. 5), suggesting that all of these mono-, di-, and tricarboxylic acids used the same low-affinity citric acid transport system. We concluded that citric acid-grown cells of the yeast *C. utilis* induce two transport systems for citric acid, presumably a proton symport and a facilitated diffusion system for the charged and undissociated forms of the acid, respectively. Both systems could be investigated by manipulating the pH and, as a consequence, the relative concentration of the anionic and undissociated forms of the acid. While the facilitated diffusion function was absent or not measurable at pH values higher than 5.0, the facilitated diffusion function coexisted with proton-citrate symport activity when the uptake was measured at low pH values (from pH 4.0 to pH 2.0). The specificity of this low-affinity transport system for other substrates and the question of whether the system is a general carboxylic acid permease are under study.

Regulation of the two citric acid transport systems. Cells of *C. utilis* grown in a medium with either ethanol (0.5%, wt/vol) or glycerol (0.5%, wt/vol) as the carbon source did not transport citric acid in the pH range between 3.0 and 6.0, indicating that both citric acid carriers were inducible. Growth of the yeast cells in a medium containing glucose (0.1%, wt/vol) and citric acid (0.5%, wt/vol) was diauxic (Fig. 6). Activities of high- and low-affinity systems as well as citric acid assimilation became detectable only after glucose had been consumed, suggesting that both transport systems were inducible and subject to glucose repression.

Growth of the yeast in a medium with L-lactic acid or L-malic acid as the carbon source also resulted in high- and low-affinity citric acid transport activities. These results suggested that not only citric acid but also other carboxylic acids act as inducers of the citric acid transport systems.

The high- and low-affinity citric acid transport systems were also found in cells that had been grown in citric acid (0.5%, wt/vol) medium at pH values lower or higher than 5.0 as described in Materials and Methods. The results showed that even the cells grown either at the lowest relative anionic-form concentration (pH 2.0) or in the absence of the undissociated citric acid (pH > 6.0) displayed a biphasic Lineweaver-Burk plot with a low-affinity component such as that in Fig. 4, when the uptake was measured at a pH lower than 5.0 and a linear Lineweaver-Burk plot over the whole concentration range at uptake pH values higher than 5.0, such as that in Fig. 1. We concluded that the induction of both systems, the proton symport and the facilitated diffusion system, was not dependent on the relative concentration of an anionic form(s) and of undissociated citric acid in the culture medium.

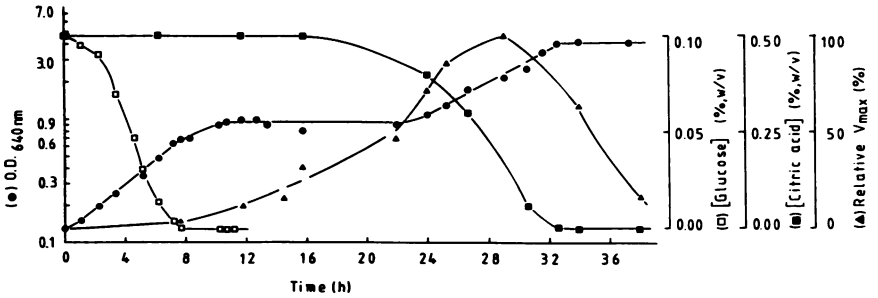

FIG. 6. Growth of *C. utilis* IGC 3092 at pH 4.8 in a mineral medium with vitamins, glucose (0.1%, wt/vol) and citric acid (0.5%, wt/vol). $O.D._{640}$ nm, optical density at 640 nm; Relative V_{max}, relative activity of the proton-citrate symport measured at a saturating concentration of citric acid (3.9 mM); w/v, weight/volume.

Simple diffusion of undissociated acid. Glucose-grown cells lacking the citric acid carriers were still slightly permeable to the acid. Plots of the initial uptake rates of citric acid against the concentration of undissociated acid were linear (Fig. 7), indicating simple, non-carrier-mediated diffusion. Estimates of the diffusion constants were obtained from the slopes of the linear plots at the various pH values. The diffusion constant, expressed in the units of the plots of Fig. 7, has the dimensions of volume (microliters), reciprocal time ($seconds^{-1}$), and reciprocal biomass ($milligrams^{-1}$). The values of the diffusion constant decreased steeply with the extracellular proton concentration from about 1 at pH 5.0 to 0.01 at pH 2.5 (Fig. 7, insert). As a consequence, the passive diffusion of undissociated citric acid across the plasma membrane of *C. utilis* is subject to opposite pH influences: an increase due to the relative increase of undissociated acid with decreasing pH and a decrease due to decreasing permeability with decreasing pH. Similar behavior was observed earlier with respect to passive proton diffusion across the plasma membrane of *Saccharomyces cerevisiae* (7), passive diffusion of undissociated lactic acid across the plasma membranes of *C. utilis* and *S. cerevisiae* (2, 8), and passive diffusion of undissociated malic and succinic acids across the plasma membrane of *C. sphaerica* (4) and *Hansenula anomala* (3), respectively.

When the results are analyzed as a whole, the comparison of the relative values of uptake rate in citric acid-grown cells and in glucose-grown cells is firm evidence that we are dealing with a carrier-mediated transport in the case of citric acid-grown cells. As for the mechanism involved, the argumentation based on the values for K_m for the high-affinity system calculated under the assumption that the undissociated or the anionic form(s) is transported is a good basis to predict that an H^+-citrate symport is involved. Further studies are needed to elucidate the proton-citrate stoichiometry and the question of which citrate ions are transported by the symporter. The basis for our assumption that the low-affinity system consists of facilitated diffusion of the undissociated acid is the relatively poor inhibition caused by CCCP. A better understanding for the mechanisms involved will certainly be achieved with a study based on vesicles prepared from cells grown under various conditions.

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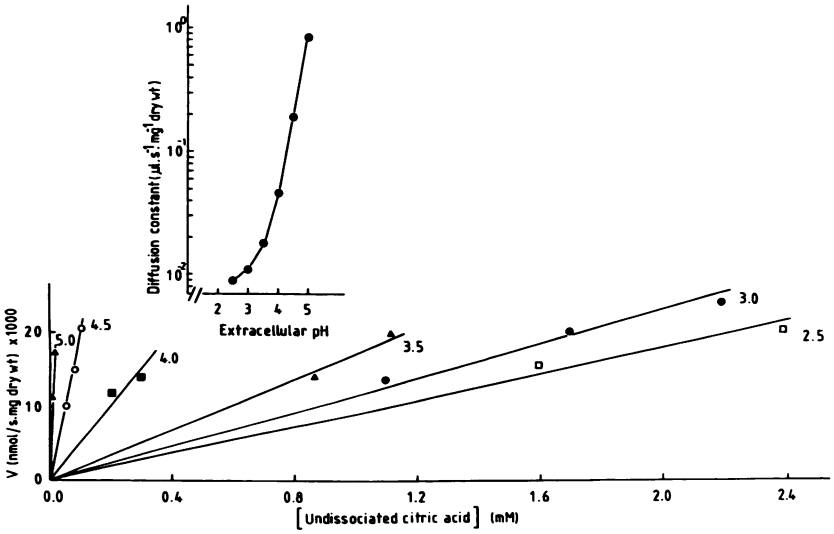

FIG. 7. Initial rates of uptake of undissociated citric acid, as a function of its concentration, by glucose-repressed cells of *C. utilis* IGC 3092. Numbers at the ends of lines connecting datum points indicate pH values. (Insert) pH dependence of the diffusion constants calculated from the slopes.

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