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FACTORS GOVERNING SUBSTRATE-INDUCED GENERATION AND EXTRUSION OF PROTONS IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Summary

Experiments with respiration deficient (ρ^-), ADP/ATP transport deficient (op_1) and double ($op_1\rho^-$) mutants, with glycolytic and tricarboxylic acid cycle substrates showed that the substrate-induced acidification of yeast suspensions is closely associated with glycolysis. The glucose/proton stoichiometry is 2.5 : 1 to 4 : 1 depending on glucose concentration. The kinetics of the process are complex, the acidification curve having a very fast initial component and two slower exponential components. The first component suggests an initial proton efflux from endogenous sources, triggered by exogenous substrates. The acidification process exhibits two K_m values at about 1 and 15 mM D-glucose, indicating two distinct saturable pathways of proton extrusion. The total extent of acidification and thus the final pH_{out} reaches a saturation value with increasing glucose concentration and suspension density. Both the total extent and the rate of acidification are subject to control by extracellular pH which reflects the tendency of the cells to build a fixed $[H^+]_{out}/[H^+]_{in}$ ratio. When the control is lifted, both quantities are considerably increased. A crucial role in the substrate-induced acidification is thus played by active membrane processes and their control mechanisms.

Introduction

An acidification of outer medium after addition of suitable substrates has long been observed in suspensions of the longest-known cultural yeast, *Sac-*

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide.

charomyces cerevisiae, but it is known to occur also in other yeast species. In spite of its being known in brewing and distillery industries for decades or perhaps centuries, the purpose of the process is still not quite clear. It may be associated with the secondary active transport of nutrients such as sugars, amino acids or purines [1—3] at the expense of energy stored in the electrochemical gradient of protons formed across the plasma membrane. It may be connected with the production of intracellular acids and their extrusion from cells upon the addition of substrate. The extrusion may help in eliminating excessive acidity from the cell interior and may create a pool of extracellular metabolites utilized by the cells after the added substrate has been exhausted. Another purpose may be the creation of a constant buffering capacity of the external medium [4] which may to some degree resemble the homeostasis in multicellular organisms.

The molecular nature of the process is not yet known in detail, the recently described plasma membrane ATPase [5—11] may be a candidate but in fact two or more mechanisms can be involved.

Attempts to elucidate some of these points are described in this paper.

Materials and Methods

The strains used included commercial pressed baker's yeast, an aneuploid strain *Saccharomyces cerevisiae* K derived from distillery yeast, *S. cerevisiae* R XIIA, *S. cerevisiae* Σ 1278b, and mutants R XIIA ρ^- (respiration deficient), op_1 (defective in mitochondrial ADT/ATP transport) and a double mutant $op_1\rho^-$. All strains were from the collection of this laboratory, the op_1 and $op_1\rho^-$ mutants being obtained from Dr. J.Šubík of Bratislava.

Strains were maintained on agar slopes and grown in flasks on a reciprocal shaker at 28°C to the stationary phase in a synthetic medium containing ($g \cdot l^{-1}$): KH_2PO_4 2.0, K_2HPO_4 1.0, $(NH_4)_2SO_4$ 2.0, trisodium citrate 0.3, $MgCl_2 \cdot 6\,H_2O$ 0.1, $MnSO_4 \cdot 7\,H_2O$ 0.1, $CaCl_2$ 0.1, $ZnSO_4 \cdot 7\,H_2O$ 0.1, yeast extract 3.0, pH 5.5—6.0. Aliquots of 100 ml in 500-ml flasks were supplemented with 2 ml sterile 40% glucose solution prior to inoculation; other growth substrates included galactose, maltose and glycerol. The harvested cells were washed by centrifugation, aerated for 3 h to deplete them of endogenous substrates, and used for experiments; the same washing and starvation procedure was used with pressed baker's yeast. Occasionally, the cell pellet was left overnight in a refrigerator at 4°C and used on the following day; this had no effect on the results.

For experiments the cells were suspended in distilled water to the required density.

pH measurements were done on a digital pH-meter, model M 120 (Development Workshops, Czechoslovak Academy of Sciences) with a recorder. The suspension volume was 9 ml unless otherwise stated; all measurements were done in a constant-temperature bath at 30°C.

pH-stat measurements were done on a Radiometer Copenhagen pH-stat using a Radiometer 2320C combined glass/calomel electrode.

Acidification was induced in all cases by adding an appropriate amount of substrate. Aeration of yeast suspension was ensured by magnetic stirring; if

necessary, anaerobic conditions were imposed by 1-min thorough flushing of the suspension with argon from a cylinder prior to measurement and by continuous blowing of the gas (containing about 0.4 ppm O_2) on the surface of the suspension during acidification. Measurements in air and in argon were usually done in close succession to ensure adequate comparison.

The constant temperature, as well as the constant, carefully adjusted stirrer frequency employed in all experiments served to eliminate variations in the response of the electrode couple due to temperature changes, varying liquid-junction potential, possible suspension effect caused by cell sedimentation, varying amounts of gases (O_2, CO_2) dissolved in the yeast suspension, etc.

The actual total amount of extruded protons, A_a, was calculated from the difference in suspension pH before and after acidification; the corresponding net H^+ fluxes, J_a, were obtained from the slopes of recorded acidification curves. The total extent of acidification, A_s, and corresponding H^+ fluxes, J_s, measured at constant pH_{out} were obtained from pH-stat measurements. The pH of the suspension was kept constant at a preselected point on the acidification curve by continuous addition of 0.1 M NaOH and the flux was calculated from the extrapolated initial slope of the recorded NaOH consumption curve.

For the calculation of proton fluxes the area of the yeast plasma membrane was expressed in cm^2/mg dry weight. The cells of *S. cerevisiae* may be represented by prolate ellipsoids; micrometric measurements showed that the mean axis lengths were 7.67 ± 1.60 μm and 6.08 ± 1.24 μm (n = 25, mean ± S.E.). Assuming a 30% proportion of dry weight in the overall cell wet weight, and dry weight density of 1.15 (based on the proportion of proteins and polysaccharides), the mean surface area of the plasma membrane corresponding to 1 mg dry weight is S = 29 cm^2. Burrow-like invaginations (cf. Ref. 12) will add another 5—10% to the surface area; hence the total surface area S_{dw} is about 32 cm^2/mg dry weight. Although representing only a rough estimate of the plasma membrane area, this value is useful in converting the measured fluxes to values commensurate with those obtained in other kinds of cells.

Intracellular pH was determined by measuring the distribution of bromophenol blue between the cells and the medium [13].

Respiration and fermentation rates were determined by the direct Warburg method at 30°C.

Results

Basic features of acidification

The extracellular pH of a resting yeast suspension in distilled water ranges from 4 to 8 depending on yeast strain and previous history (starvation, storage, etc.). Addition of substrate such as D-glucose elicits a rapid pH drop of 1—4 units. In our experiments the drop was almost identical in the facultative strain *S. cerevisiae* K and in the op_1, ρ^-, and $op_1\rho^-$ mutants (Fig. 1). It was also analogous under aerobic and anaerobic conditions over a wide range of glucose concentrations (1—200 mM). On the other hand, it should be noted that *S. cerevisiae* mutants blocked in glycolysis do not exhibit acidification while being able to take up glucose, and the fact mey even be used for their detection and diagnostics [14].

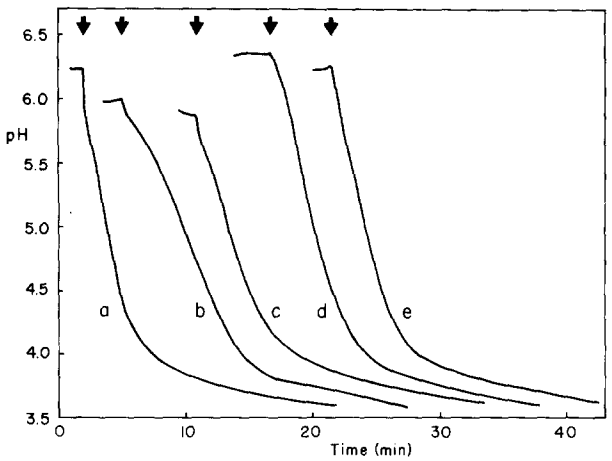


Fig. 1. Acidification curves of *S. cerevisiae* after glucose addition. Yeast dry weight 10 mg/ml, 200 mM glucose. a, *S. cerevisiae* K aerobically; b, respiration-deficient mutant ρ^-; c, ADP/ATP transport-deficient mutant op_1; d, double mutant $op_1\rho^-$; e, *S. cerevisiae* K anaerobically. Glucose addition indicated by arrow.

The acidification rate, but not the total extent, is reduced by high external osmolarity (0.5 M mannitol). The rate is also lower in cells tested immediately after growth or subsequently enriched with glucose than in starved cells, possibly corresponding to slower glucose utilisation under such conditions.

Fig. 2 lists typical acidification curves caused by diverse substrates. Trehalose and maltose are seen to cause a transient pH rise currently though to indicate proton-substrate symport [1,15,16]. On the other hand, sucrose, D-fructose, D-mannose and D-galactose (after induction) yield acidification curves essen-

TABLE I

RESPIRATION AND ACID PRODUCTION IN *SACCHAROMYCES CEREVISIAE* K ON DIFFERENT SUBSTRATES

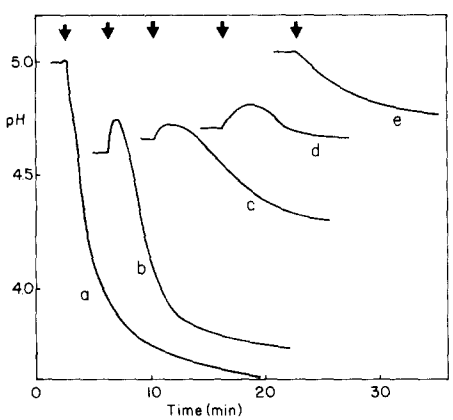
Substrate	% relative to glucose	
	Q_{O_2}	Acid extrusion
Glucose	100	100
Galactose (after induction)	37	42
Maltose	99	58
Sucrose	74	95
Fructose	108	102
Mannose	93	90
Glycerol	14	1
Pyruvate	8	0
Lactate	43	0
Ethanol	130	9
Acetate	83	0
Citrate	8	0
Succinate	5	0
Fumarate	5	0
Malate	6	0

tially identical with that caused by D-glucose. Nonmetabolized sugars transported into the cells by the constitutive specific monosaccharide transport system used by glucose [17], e.g. 3-*O*-methyl D-glucose, 2-deoxy-D-glucose, D-xylose and D-arabinose, evoke no pH change on addition to the cells (data not shown).

As shown in Table I, a fairly good correlation between acidification and respiration was found with glycolytic substrates, the discrepancy found with maltose being obviously due to its active uptake via maltose-proton symport (in contrast to the facilitated diffusion operative with the monosaccharides). On the other hand, an almost complete absence of acidification, though sometimes with intensive respiration, was found with substrates utilised below the glyceraldehyde 3-phosphate → 1,3-diphosphoglycerate reaction. Ethanol and acetate, although providing high respiration rates, caused little or no acidification.

The above findings indicate that the acidification is closely associated with glycolysis.

The proton/glucose stoichiometry was found to have no fixed value, ranging from 1 : 2.5 to 1 : 4 depending on glucose concentration. This may reflect the actual proportion of added glucose that serves as source of protons (related to the level of aerobic glycolysis), relative efficiency of glucose uptake and dissimilation systems as compared with those responsible for proton generation



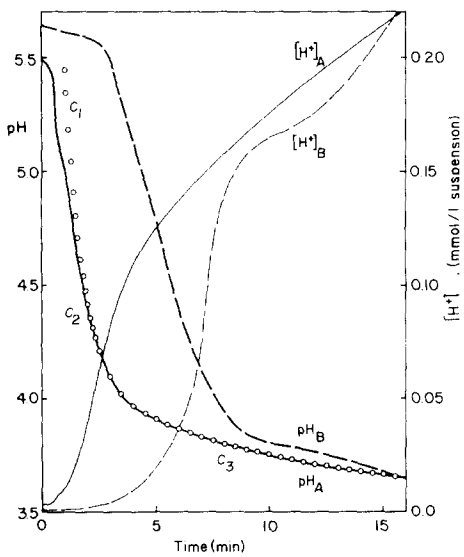


Fig. 2. Typical acidification curves of *S. cerevisiae* K with different substrates. Yeast dry weight 10 mg/ml, 50 mM substrates, a, D-glucose, D-mannose, D-fructose, D-galactose (after 2.5-h induction); b, maltose after 2.5 h induction; c, maltose without induction; d, trehalose; e, ethanol. Substrate addition indicated by arrow.

Fig. 3. Characteristics of acidification curves of wild-type (A) and respiration-deficient (B) cells of *S. cerevisiae*. Yeast dry weight 10 mg/ml, 100 mM glucose. pH_A, acidification curve of A; points indicate exponential function fitted to experimental curve; $[H^+]_A$, corresponding proton concentration curve. pH_B, acidification curve of B; $[H^+]_B$, corresponding proton concentration curve. C_1, C_2, C_3, segments of pH_A described in the text. Glucose added at $t = 0$.

and extrusion, or the action of membrane processes controlling proton efflux (see below).

Typical acidification curve

The typical acidification curve observed after the addition of glucose is shown in Fig. 3. The length of the lag observed sometimes between glucose addition and the onset of pH drop depends on the extent of cell starvation, disappearing in cells starved for 3 h or more. The lag is followed by a rapid pH drop (curve segment C_1) which is interrupted by a slight shoulder. An initial drop and a shoulder, though not of the same extent and duration, were found also on the glucose consumption curve at glucose concentrations higher than about 100 mM [18] and on the intracellular level of inorganic phosphate after glucose addition [19]. At pH values below about 4.5 the acidification curve may be described by the sum of two exponentials, no other fits (parabolic, hyperbolic, etc.) being satisfactory. In the case depicted in Fig. 2,

$$\mathrm{pH}(t) = C_2 + C_3 = 3.55 + 0.628\, e^{-0.115t} + 5.562\, e^{-1.359t} \quad (1)$$

with coefficients of correlation $r(C_2) = 0.997$ and $r(C_3) = 0.999$. The approximately 10-fold difference between the coefficients of C_2 and C_3 suggests an efflux from two compartments, or the presence of two transport systems, with widely different properties. Component C_2 virtually disappears at pH values below about 3.9.

If the progress of acidification is expressed in terms of extracellular concentration of protons, one may observe a steeper increase after an initial period of 4—6 min. This may correspond to the start of the H^+-extrusion system or to gradually increasing availability of protons inside the cells.

In some cases of perturbation of cell processes, such as the application of inhibitors (nystatin, iodoacetamide, fluoride, fluoroacetate, 2,4,6-trinitrophenol, CCCP, azide, DCCD) in concentrations which do not yet affect the total extent of acidification and metabolism [20] or when acidification is induced, e.g. by glucose in glycerol-grown yeast, as well as in the ρ^- mutant, the C_3 component is altered and a second shoulder appears on the $[H^+]$ curve (Fig. 3). In yeast grown on galactose and maltose the acidification caused by glucose is essentially identical with that found in glucose-grown yeast (data not shown).

Factors governing the total extent of proton extrusion

The dependence of the actual total proton extrusion, A_a, on glucose concentration (Fig. 4) and yeast suspension density (Fig. 5) has a saturation character. The maximum values attained at suspension dry weight higher than about 10 mg/ml and glucose concentrations higher than 20—30 mM range from 200 to 800 μmol H^+/l suspension. For a given yeast batch this value, and consequently the lowest attainable pH_{out}, is a characteristic constant. This seems to indicate that the cells tend to attain a fixed $[H^+]_{out}/[H^+]_{in}$ ratio (10^2—10^3 depending on yeast batch and experimental conditions). Moreover, the transient drop in intracellular pH observed in most experiments after glucose addition (Fig. 6; see also Ref. 21) diminishes with increasing suspension density. Both the net outflow of protons from the cells and their production in the cells (or perhaps

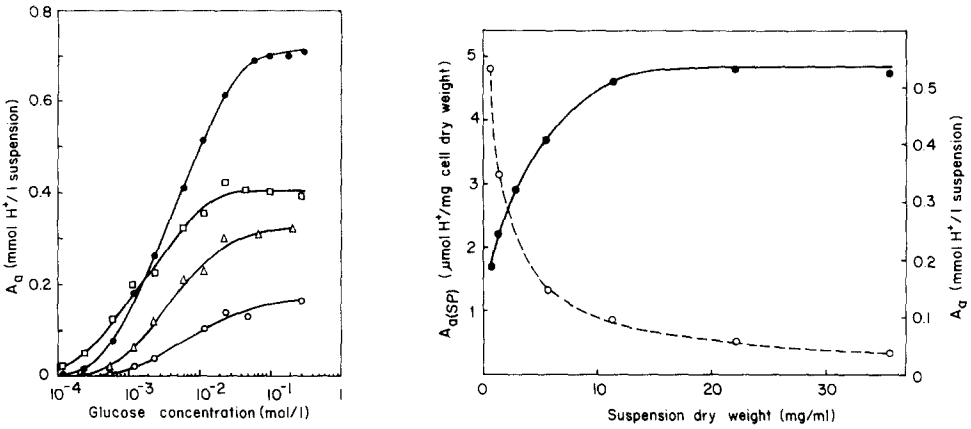


Fig. 4. Dependence of total proton extrusion A_a in *S. cerevisiae* strains on glucose concentration. Yeast dry weight 10 mg/ml. ●———●, *S. cerevisiae* K; □———□, baker's yeast; △———△, *S. cerevisiae* Σ 1278b; ○———○, *S. cerevisiae* R XIIA.

Fig. 5. Effect of suspension density on total (A_a) and specific ($A_{a(SP)}$) actual proton extrusion in *S. cerevisiae* K. 40 mM glucose. ●———●, A_a; ○———○, $A_{a(SP)}$.

the uptake or utilization of the substrate) thus seem to be lowered due to the high extracellular concentration of protons created in the suspension (cf. below).

The total extent of acidification is apparently also subject to control by low extracellular pH: when the pH_{out} was prevented from decreasing after glucose addition (i.e. on a pH-stat), the extent of acidification increased 1.5- to 7-fold depending on yeast dry weight [25].

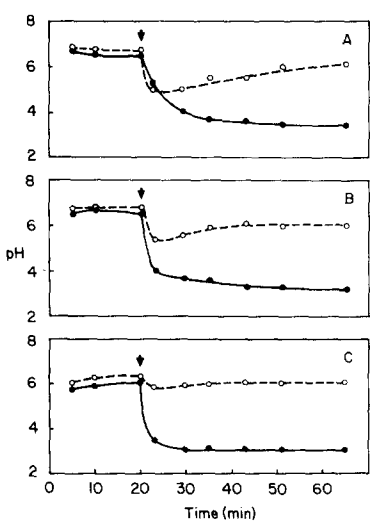


Fig. 6. Effect of suspension density of extra- and intracellular pH in *S. cerevisiae* K supplied with 100 mM glucose. A, 4% suspension; B, 10% suspension; C, 20% suspension. ●———●, pH_{out}; ○- - - - -○, pH_{in}. Glucose addition indicated by arrow.

Factors governing net proton fluxes

The dependence of J_s, the net specific proton efflux measured by pH-stat at constant pH_{out}, as well as J_a, the actual net proton efflux calculated from initial slopes of acidification curves, on glucose concentration has a saturation character in all strains tested. A Lineweaver-Burk plot of J_s versus glucose concentration yields two K_m values about an order of magnitude apart (Table II) and two corresponding values of J_{max}. Similar K_m values are obtained with J_a although the values of J_{max} are about 100 times lower. The apparent two distinct saturable pathways of proton extrusion may, or may not, be related to the presently recognized plurality of glucose uptake systems in baker's yeast, constitutive nonspecific plus constitutive specific system [17] as well as some of the disaccharide transport systems [16].

The effect of suspension density on J_s and J_a is shown in Fig. 7. The much higher J_s does not vary with suspension dry weight while J_a markedly decreases as the suspension dry weight is increased to about 10 mg/ml and then it levels off. The dependence is shown for both the C_1 and C_2 components of the acidification curve. The graph points to the role of extracellular acidity in the regulation of proton fluxes. When the extracellular pH is kept constant, i.e. in pH-stat experiments, the regulation is abolished and J_s is a constant irrespective of the number of cells in the suspension while in an unbuffered suspension a higher number of cells causes a more rapid drop of pH_{out} and thus a reduction of J_a.

A similar conclusion can be drawn from Fig. 8 which shows the dependence of J_s and J_a on pH_{out}. The difference between J_s and J_a curves reflects the disparity between the specific net proton effluxes observed under the two different sets of conditions, i.e., when the cells are allowed to build up their characteristic $[H^+]_{out}/[H^+]_{in}$ ratio (J_a), and when this spontaneous trend is disturbed by maintaining the ratio artificially lower than would be attained by cells (J_s). The difference diminishes with decreasing pH, reflecting the diminishing difference between the spontaneously attained and artificially maintained $[H^+]_{out}/[H^+]_{in}$ ratio. The increase in J_a values between pH 5.5 and 4.0 may correspond to the initial 'sigmoid' phase of the $[H^+]$ curves in Fig. 3.

TABLE II

K_m and J_{max} VALUES OF PROTON EXTRUSION MEASURED BY pH-STAT (J_s) AND CALCULATED FROM SLOPES OF ACIDIFICATION CURVES (J_a) IN VARIOUS STRAINS OF *SACCHAROMYCES CEREVISIAE*

Suspension dry weight 10 mg/ml; measurement at pH 5.5.

Strain	$K_{m(1)}$ (mM)	$K_{(m(2)}$ (mM)	pmol · cm^{-2} · s^{-1}			
			$J_{s(max)}$		$J_{a(max)}$	
			1	2	1	2
Commercial baker's yeast	1.4 *	17.2 *	17.8	52.6	0.19	0.58
S. cerevisiae K	1.3	11.8	43.4	74.0	—	—
S. cerevisiae 1278b	0.8	13.5	13.0	38.4	—	—
S. cerevisiae RXII	1.5	15.4	58.4	116.2	—	—

* Calculated from pH-stat measurements.

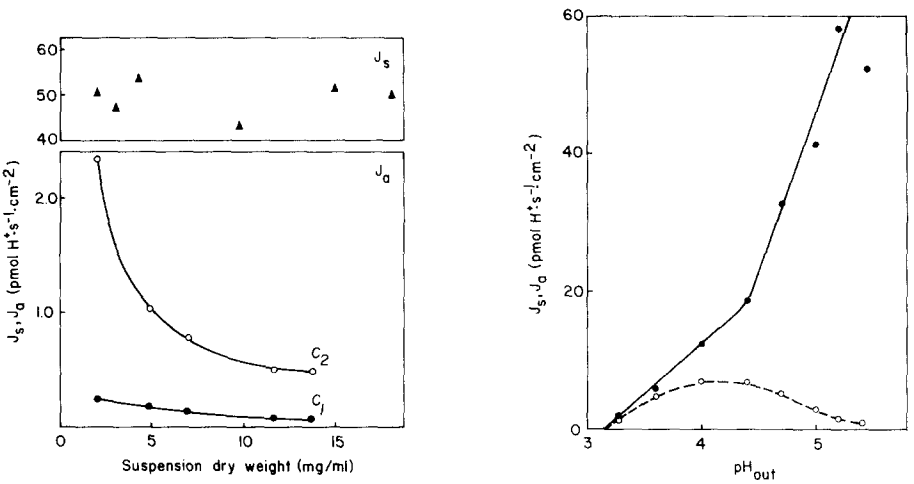


Fig. 7. Effect of suspension density on pH-stat (J_s) and actual (J_a) net specific proton efflux in *S. cerevisiae* K. 40 mM glucose. Values obtained at pH_{out} 5.8—6.0 (J_s), 5.5 (J_a-C_2) and 7.0 (J_a-C_1).

Fig. 8. Effect of extracellular pH on pH-stat (J_s) and actual (J_a) net proton efflux. Yeast dry weight 10 mg/ml, 22 mM glucose. ●———●, J_s; ○- - - - - -○, J_a.

Discussion

The obvious conclusion that can be drawn from our experiments with different mutants of *S. cerevisiae* and with different acidification-inducing substrates as well as from previous studies [20] is that for the most part the extruded protons arrive from glycolysis. The acidification process may be envisaged as a simple chain of events: uptake of glycolytic substrate → enzymic conversion of the substrate into acid metabolite(s) → extrusion of (surplus) acidity from the cell. The possibility that the substrate uptake itself can be associated with extrusion of protons by an exchange mechanism has been excluded by our experiments with nonmetabolizable sugars transported into the cell by the glucose-transporting system.

The above picture is supported by our finding of a transient decrease in intracellular pH after glucose addition. This agrees with the similar, though less pronounced, transient intracellular acidification found by ^{31}P-NMR spectroscopy [21]. (The alkalization sometimes found [22,23] may reflect different experimental conditions and also a difference in the relative activity of H^+-producing and H^+-extruding systems in yeast strains under study). This decrease suggests the generation of net acidity in the cells during acidification. In the simplest case the extrusion of acid metabolites could proceed passively down their concentration gradient and the whole process would represent a means whereby the cells eliminate excessive intracellular acidity. The actual acidification process is much more complex. No specific data are as yet available on the transport mechanisms of its various components but the acidification appears to involve a passive extrusion of metabolites such as CO_2, glycerol and organic acids [18] or their anions which can act as counterions to excreted

cations (cf. the following paper [25]). However, our previous study [20] showed that it also includes an active membrane process, most probably extrusion of protons, which can be blocked by a number of inhibitors.

The complex acidification kinetics observed in this study suggest the presence of several active transport systems. Furthermore, the high rate and immediate onset of the C_1 component of the acidification curve, especially in starved cells, seems to indicate that the uptake of glucose triggers a rapid and immediate extrusion of protons from endogenous sources. A similar boost of endogenous processes by exogenous substrate is described elsewhere [24].

The apparent 'cooperative' character of initial phases of the C_2 segment of acidification curve can reflect the actual beginning of glucose utilization, but may also mirror the start-up of another transport process. The data available on the initial phases of glucose dissimilation do not permit us yet to decide between the alternatives.

Our results show that both the extent and the rate of acidification are controlled by external pH. The lifting of this control causes a 10- to 100-fold increase in proton efflux and a large increase in acidification extent and is also responsible, e.g. for the increased proton fluxes observed in buffered suspensions [9]; the increase in the proton flux cannot be due to the action of buffer molecules at the membrane as proposed by Serrano [9] since it occurs even in the absence of any added buffer. The control by external pH reflects the tendency of the cells to create a fixed $[H^+]_{out}/[H^+]_{in}$ ratio. The control can be exerted on several levels: (1) Low pH_{out} can inhibit the uptake of glucose. This mechanism can be expected to play a significant role near pH_{out} 3 as the glucose transport system was reported to have a broad pH optimum (4—6; cf. Ref. 26). (2) The controlled step may be the actual formation of the source of acidity inside the cells as indicated by the diminishing of transient decrease in pH_{in} after glucose addition to suspensions with increasing density. (3) The low pH_{out} may inhibit membrane-located proton extrusion systems. This possibility is borne out by the rapidity of response of the cells to a challenge with a large dose of alkali [25]. Further studies are required to determine the exact mechanism of the pH_{out} control.

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