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# Energetics of L-proline uptake by *Saccharomyces cerevisiae*

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**L-Proline is transported into the yeast *Saccharomyces cerevisiae* against a concentration gradient of up to 135:1, the gradient decreasing with increasing proline concentration and suspension density. The concentrative uptake is practically unaffected by inhibitors, except antimycin. It is markedly reduced by anaerobic conditions. Uptake of L-proline, either by normal cells or in the presence of inhibitors, elicits no alkalification of the medium (estimated by pH and conductivity measurements) and no membrane depolarization (estimated by distribution of tetraphenylphosphonium). There is no relationship between the electrochemical potential gradient of protons and the measured accumulation ratios of proline. Likewise, intracellular ATP levels bear little relation to the accumulation. If, based on analogy with other yeasts and bacteria, L-proline is symported with $H^+$ ions the process must occur in local domains of the membrane where both the $\Delta pH$ and the membrane potential may differ substantially from those measured in the bulk solution.**

## Introduction

An earlier paper [1] described the kinetics of L-proline uptake by baker's yeast through a high-affinity ($K_T = 31$ $\mu$M) system which, in contrast with other natural amino acids, appears to be able to operate reversibly so that proline appears in the external medium when proline-loaded cells are transferred to a proline-free medium.

The accumulation of L-proline in baker's yeast cells and the possible source of energy for this process are considered in the present paper.

## Materials and Methods

*Microorganism.* The facultatively anaerobic aneuploid strain *Saccharomyces cerevisiae* K (CCY 21-4-60) was used throughout. Cells were grown in flasks on a reciprocal shaker (2 Hz) at 30°C to early stationary phase (20–22 h) in a semisynthetic medium containing L-proline (1 mg/ml) as the sole source of nitrogen [1]. Harvested cells were washed in tap water, aerated on a magnetic stirrer for 2 h and the pellet was left to stand at 4°C overnight.

*Initial rate and accumulation ratio measurements.* After a 1-h incubation with 1% glucose which stimulates the synthesis of the specific L-proline transport system [2], the yeast cells (15–25 mg dry weight/ml) were washed with distilled water and resuspended in 0.2 M triethanolamine/phthalate buffer (0.2 M triethanolamine was adjusted to the appropriate pH with 0.2 M phthalic acid) in Erlenmeyer flasks at 30°C in a Dubnoff incubator. After 10 min of preincubation with 0.4 mM cycloheximide, $^{14}$C-labelled L-proline was added to 0.1 mM. Intracellular concentration of L-proline was determined after filtration of 0.2 ml suspension through Synpor 5 (0.6 $\mu$m pore diameter) filters. The cell pellet on the filter was twice washed with 1 ml ice-cold buffer and transferred to a scintillation vial with 10 ml toluene + ethanol scintillation cocktail. Extracellular radioactivity was determined by counting an aliquot of the

supernatant after centrifugation in an Ecco-Quick centrifuge. For accumulation ratio calculations, the value of 2.21 $\mu$l intracellular water volume per mg dry weight was used [3].

*Determination of $\Delta pH$ and transmembrane potential.* $\Delta$pH and $\Delta\psi$ were determined from the accumulation of 5 $\mu$M $^{14}$C-labelled propionic acid and 3–5 $\mu$M $^{3}$H-labelled tetraphenylphosphonium bromide, respectively [4,5]. The yeast suspension was prepared in the same way as for the transport studies.

*Measurements of $pH_{out}$ changes.* These were done in an unbuffered or lightly buffered (1 mM triethanolamine/phthalate) yeast suspension at 30°C in 40 ml constant temperature, magnetically stirred vessels, using a PHM 83 Autocal Radiometer with an appropriate combined glass-calomel electrode.

*Conductometry.* The conductivity of cell suspensions was monitored in the presence of various substrates and inhibitors using a locally made device consisting of two platinum electrodes, connected with a Wheatstone bridge fed with a current of 300 Hz at which cells are unaffected and do not move in the alternating field. Changes of conductivity were recorded as differences in output voltage of the bridge.

*Determination of intracellular ATP content.* Yeast cells (5–9 mg) were extracted with 0.4 M perchloric acid for 10 min in the cold in a final volume of 2 ml. After centrifugation 0.5 ml of the supernatant was adjusted to pH 6 with a solution containing 1 M NaOH and 0.2 M $NaHCO_3$. After 5 min in the cold it was centrifuged and the supernatant was used for ATP determination by the luciferin-luciferase method [6].

*Chemicals.* All compounds were of the highest available purity. They included diethylstilbestrol, 3-chlorophenylhydrazonomalononitrile (CCCP), L-proline and luciferin-luciferase (Sigma, Switzerland), cycloheximide (Upjohn, U.S.A.), *N,N'*-dicyclohexylcarbodiimide and antimycin (Serva, FRG). The remaining compounds were obtained from Lachema (Czechoslovakia). Carrier-free $^{14}$C-labelled propionic acid (1.85–2.2 GBq/mmol) and $^{3}$H-labelled tetraphenylphosphonium bromide (0.74–1.5 TBq/mmol) were from Amersham International (U.K.). Uniformly $^{14}$C-labelled L-proline was from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia).

## Results and Discussion

*Accumulation ratio of proline*

The final accumulation ratio of L-proline was found to decrease with increasing L-proline concentration, a common observation with actively transported solutes (Fig. 1). In principle, such a decrease may be due to two causes: (1) a parallel diffusion or mediated diffusion leak which becomes prevalent at high concentrations (cf. Ref. 7); (2) the relative unavailability of the source of energy at high proline concentrations (cf. Ref. 8). Although a leak of proline was assumed to exist together with the active system [1] the results in Fig. 1, specifically the decrease of the $s_{in}/s_{out}$ ratio below unity, is fairly strong evidence against its operation. The typical shape of the curve resembles that predicted in Ref. 8 for a situation where coupling with energy is obligatory and where the sequence of partial transmembrane steps is such that the source of energy interacts only with the carrier-solute complex but not with the free carrier (either model I or III of the paper cited). An analogous situation was described in Ref. 9 for L-arabinose concentration in *Rhodotorula glutinis*

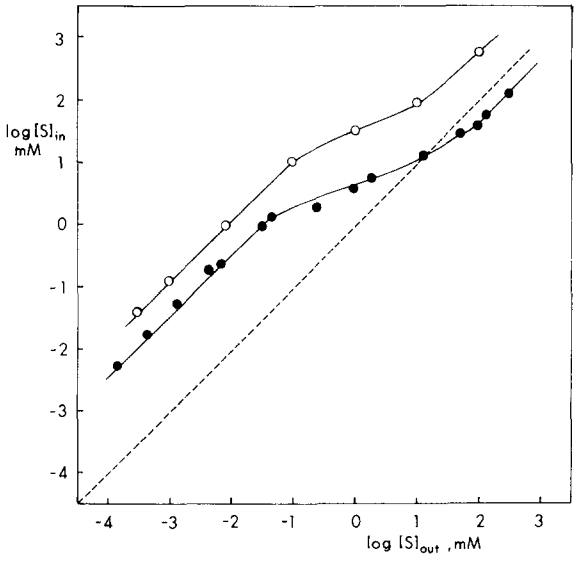


Fig. 1. Maximum intracellular concentration of L-proline (ordinate) in dependence on the extracellular concentration (abscissa). The solid points were obtained at a suspension density of 18.5 mg dry weight/ml, the open points at 0.3 mg dry weight per ml.

where, however, one could conclude that the sequence of occupation of the carrier was carrier + source of energy + solute.

To be sure, the accumulation ratios of proline observed at its high concentration are not attained solely by the high-affinity system studied here. The general (?) amino acid permease responsible for proline transport at higher concentrations (its $K_T$ for proline being in the range of mM) with its 4-fold higher transport capacity will certainly contribute to it very substantially. However, whether this is the effect of one or two transport systems of proline, none of them may contain even an internal leak (i.e., transport in the absence of energization) – if it did, the final accumulation ratio would have to be always more than or equal to unity. In the presence of inhibitors, and especially anaerobically, the accumulation ratio was decreased even farther below unity when a high concentration of L-proline was used – in other words, the obligatory coupling with energy was not released by inhibitors.

In view of the remarkable effects of cell suspension density (apparently due to dissolved $CO_2$) [10] it was made sure that the dry weight concentrations used here (about 15–25 mg/ml) were well above the range where unexpectedly high metabolic rates were observed. In an experiment where a density of 0.2 mg dry weight per ml was used, the accumulation ratio of added L-proline was 135 : 1 at the lowest and 6 : 1 at the highest proline concentration used, i.e., 3- and 20-times, respectively, more than in the 'usual' dry weight range.

*Coupling with protonmotive force?*

Since proline was included by Seaston et al. [11] among amino acids eliciting a transient alkalification of the outside medium as it was added to a yeast suspension, it was examined whether the electrochemical potential gradient of protons $\Delta\tilde{\mu}_{H^+}$ (or the protonmotive force $= \Delta\tilde{\mu}_{H^+}/F$) was adequate for the accumulation ratios observed. Assuming a 1 : 1 stoichiometry between proline and $H^+$, the maximum ratio that could be achieved under tight energy coupling [12] is given by $s_{in}/s_{out} = \exp(\Delta\tilde{\mu}_{H^+}/RT)$. If the values of $\Delta$pH and $\Delta\psi$ as measured here are reliable, the energy available is adequate in the absence of inhibitors

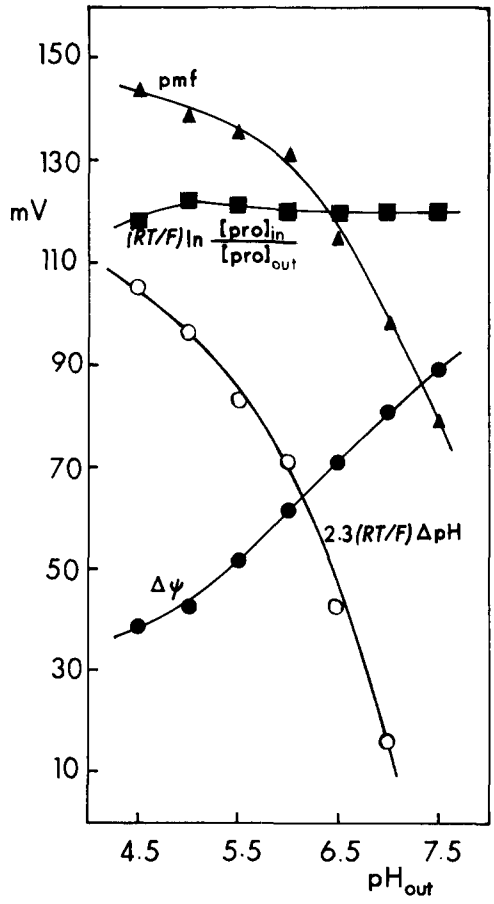


Fig. 2. pH dependence of the protonmotive force (pmf), the membrane potential $\Delta\psi$, the $H^+$ concentration term, 2.3 $(RT/F)$ $\Delta$pH, and the experimental proline accumulation, all expressed in mV.

and under normal physiological conditions at pH below 6.5 (Fig. 2). The accumulation ratio is remarkably constant (at about 65–68), this contrasting with the accumulation of sugars in *Rh. glutinis* where a peak at pH 6 was observed [13].

Temperature has little pronounced effect on the accumulation ratio, there being a basic dissimilarity with the effect on membrane potential, reflected in the protonmotive force (Fig. 3).

In the presence of inhibitors, both of transport and of metabolism, the accumulation ratios did not always lie within the range predictable from $\Delta\tilde{\mu}_{H^+}$ (Table I), notable exceptions being with CCCP and with arsenate. Strictly speaking, all cases where the last column values are above unity lie outside the permissible range. At variance with

[4] CCCP decreased especially the membrane potential, apparently by permitting $H^+$ to flow back into cells. Arsenate, especially at higher pH, decreased the $\Delta pH$ substantially, possibly by decreasing the production of ATP. This then was unavailable for the H-ATPase so that $H^+$ ions could accumulate in cells. The effects of antimycin may have been of the same origin. However, a distinction between the effects of arsenate and of antimycin is in the fact that in the presence of arsenate the accumulation of proline is normal (in fact slightly higher than in the control), while in the presence of antimycin it drops practically to zero.

Under anaerobic conditions (Table II), especially in a pH 7 buffer, the decrease of the protonmotive force represents only about 25% as yeast cells are incubated in the absence of oxygen, while accumulation of proline drops by an order of magnitude. An even more pronounced decrease in the accumulation ratio (down to unity) was described in Ref. 14 for glycine, aspartic acid and 2-aminoisobutyric acid. This suggests that another 'energy-yielding' factor, apart from protonmotive force, must be present to drive the transport of amino acids, including proline. However, all the values of intracellular concentration of proline must be considered with some doubt as, on the one hand, proline is probably accumulated in mitochondria to an unknown extent (cf. Ref. 2), while on the other, it is excluded from the vacuole [15].

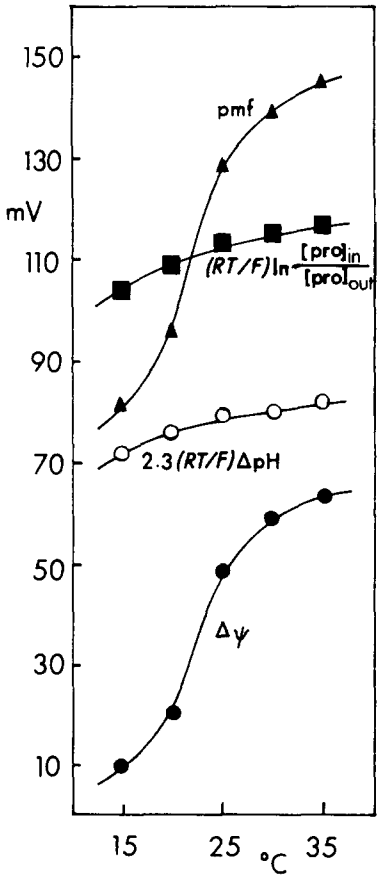


Fig. 3. Temperature dependence of the same measured values as in Fig. 2.

*pH measurement and conductometry*

The common tests for the involvement of protonmotive force in a given transport are (1) the occurrence of (transient) alkalification of the external medium as the $H^+$-symported solute is added or, if the medium is powerfully buffered, a decrease in the titration acidity, plus, in the case of yeast (cf. Ref. 11), a more or less stoichiometric

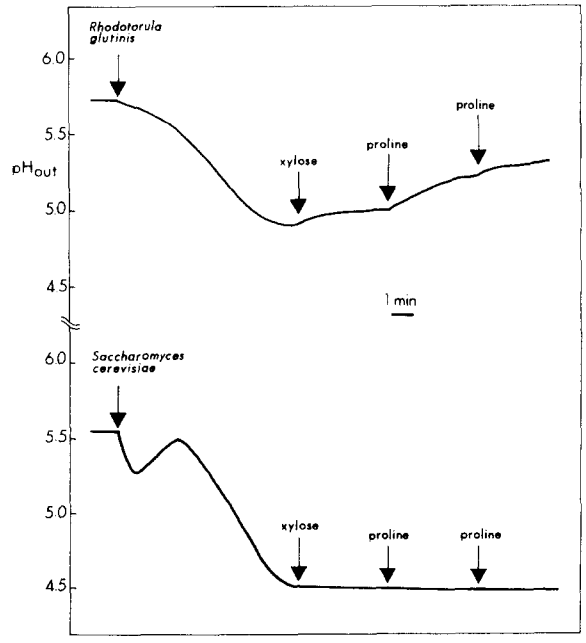


Fig. 4. Extracellular pH of suspensions of *Rh. glutinis* (5.8 mg dry weight/ml) and of *S. cerevisiae* (4.9 mg dry weight/ml) after adding D-xylose and L-proline, each to a concentration of 0.1 mM.

efflux of $K^+$ that compensates for the charge taken up in the form of $H^+$-solute complex, and (2) a depolarization of the membrane (cf. Ref. 16). In the present case, none of the tests were positive. Addition of L-proline at different concentrations to a relatively impoverished as well as glucose-preincubated suspension never evoked an alkalification. Occasionally, an acidification was observed (Fig. 4) which, according to Refs. 11 or 17, is due to an 'overshoot' of H-ATPase activity stimulated by the uptake of a $H^+$-symported solute.

Another approach, based on measuring the conductivity of suspensions, was used for comparison with the pH data. The molar conductivity of $H^+$ ions is the highest of all ions, being 373 $S \cdot cm^2 \cdot mol^{-1}$ at 30°C and for infinite dilution, followed by $OH^-$ ions (218 $S \cdot cm^2 \cdot mol^{-1}$), $K^+$ ions having a conductivity of 81 and $Na^+$ ions of 55 in the same units. The obvious disadvantage of the technique is in its nonspecificity but it has two advantages over pH measurements using a glass electrode. Its response is immediate and it registers the same change of conductivity caused by $H^+$ ions between pH 7 and 6 as between pH 3.502 and 3.501.

If there were a shift of $H^+$ ions inward during a symport process, albeit in exchange for $K^+$ coming out of cells, the conductivity should decrease, in view of the much lower molar conductivity of $K^+$. However, in the presence of salts of weak acids with strong bases in the outside medium, an opposite shift might take place. Fig. 5 shows that there is no change, even a transient one, as might be expected to occur before the ATPase is stimulated to operate. This is seen to be different from the situation in *Rh. glutinis* where proline elicits both a transient alkalification and a decrease in conductivity of the suspension.

TABLE I

EFFECT OF INHIBITORS ON MEMBRANE POTENTIAL, ΔpH AND PROLINE ACCUMULATION IN *S. cerevisiae* K

Inhibitors were added together with labelled tetraphenylphosphonium and propionic acid; after 1 h at 30°C, the supernatant was analyzed and compared with a control without cells. Means of at least four experiments are given.

| Inhibitor added | $pH_{out}$ | $\Delta\psi$ (mV) | $\Delta pH$ | pmf (−mV) [a] | Accumulation ratio, maximum theoretical [b] | Accumulation ratio, experimental | $R$ [c] |
|---|---|---|---|---|---|---|---|
| None | 4.5 | −39 | 1.78 | 146 | 271 | 64 | 0.24 |
| | 6.6 | −70 | 0.70 | 112 | 74 | 67 | 0.90 |
| Diethylstilbestrol | 4.5 | −68 | 1.61 | 165 | 562 | 62 | 0.11 |
| (50 μg/ml) | 6.6 | −93 | 0.72 | 136 | 185 | 64 | 0.35 |
| Dicyclohexylcarbo- | 4.5 | −44 | 1.33 | 124 | 117 | 65 | 0.55 |
| diimide (50 μg/ml) | 6.6 | −85 | 0.20 | 97 | 41 | 66 | 1.61 |
| 3-Chlorophenylhyd- | 4.5 | −16 | 1.04 | 78 | 20 | 67 | 3.35 |
| razonomalononitrile | 6.6 | −32 | 0.55 | 65 | 12 | 69 | 5.75 |
| (20 μM) | | | | | | | |
| Sodium arsenate | 4.5 | −33 | 1.04 | 95 | 38 | 70 | 1.84 |
| (2.5 mM) | 6.6 | −38 | 0 | 38 | 4 | 72 | 18.0 |
| KCl (0.2 M) | 4.5 | −26 | 1.79 | 133 | 165 | 62 | 0.37 |
| | 6.6 | −58 | 0.69 | 99 | 44 | 63 | 1.43 |
| 2-Deoxy-D-glucose [d] | 4.5 | −36 | 1.63 | 134 | 171 | 66 | 0.39 |
| (5 mM) | 6.6 | −65 | 0.64 | 103 | 52 | 68 | 1.31 |
| Antimycin [d] | 4.5 | −10 | 1.10 | 76 | 18 | 5 | 0.28 |
| (20 μg/ml) | 6.6 | −40 | 0.31 | 59 | 10 | 4 | 0.40 |
| Antimycin + 2-de- | 4.5 | 0 | 1.03 | 62 | 11 | 4 | 0.36 |
| oxy-D-glucose [d] | 6.6 | −34 | 0.17 | 44 | 5 | 1 | 0.20 |

[a] Protonmotive force $= \Delta\psi - (RT/F)\ln([H^+]_{out}/[H^+]_{in})$.

[b] The ratio was calculated for tight coupling with energy source as $[\text{proline}]_{in}/[\text{proline}]_{out} = \exp(\text{protonmotive force}/60)$.

[c] Ratio of experimental to theoretical accumulation.

[d] These inhibitors were added 20 min before the labelled probes.

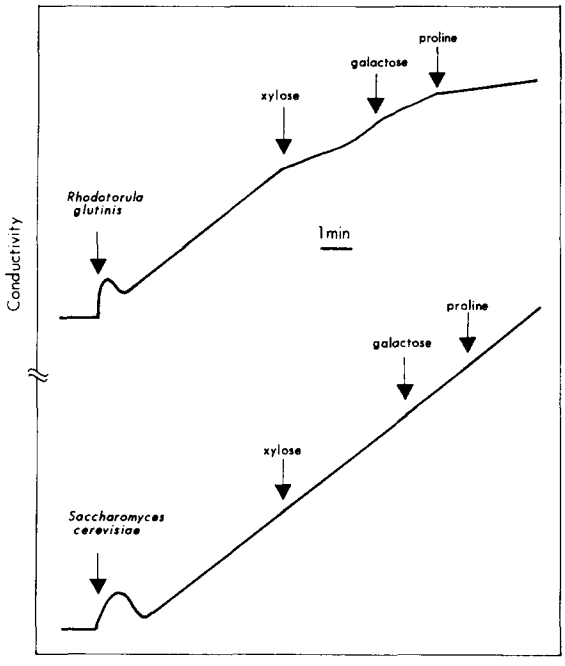


Fig. 5. Conductivity of suspensions of *Rh. glutinis* (6.1 mg dry weight/ml) and of *S. cerevisiae* (7.3 mg dry weight/ml) after adding D-xylose, D-galactose and L-proline, each to a concentration of 0.1 mM. The conductivity values are in the range of $mS \cdot cm^{-1}$.

*Other sources of energy*

The possibility was examined whether $Na^+$ ions could not be the driving ions for proline accumulation but it was found that there was a moderate depression of $\Delta\psi$ after $K^+$, $Na^+$ or $Li^+$ had been added (for $K^+$ cf. Table I) but virtually no change of proline accumulation.

In this context, the ATP levels of cells in the presence of various inhibitors were compared with the accumulation of proline (Table III). The level of ATP was affected by inhibitors in much the same way as the protonmotive force, except in the presence of dicyclohexylcarbodiimide where there was no effect on ATP but a substantial one on the protonmotive force. Except in the case of arsenate there was a good correlation between ATP levels and proline accumulation, in fact better than between the apparent protonmotive force and proline accumulation.

Thus, the present data do not permit us to make definitive conclusions about the energization of proline transport in baker's yeast. There is evidence from other cell types [11] and even from reconstituted systems [18] that amino acid uptake may be accompanied by proton translocation into cells. Indications of this were observed even here with *Rh. glutinis* (Figs. 4 and 5) and one might assume that the situation will be the same in baker's yeast at the molecular level. However, none of the classical tests bore this out and there is an overwhelming disparity, either negative or positive, between the protonmotive force computed on the basis of $\Delta\psi$ and $\Delta$pH measurements and the accumulation ratio of proline. On the one hand, accumulation ratios of 72 : 1 were found where a maximum of 4 : 1 would be expected (with

TABLE II

EFFECT OF ANAEROBIOSIS ON PROTONMOTIVE FORCE AND ACCUMULATION OF PROLINE

Cells were preincubated for 1 h with 1% D-glucose and then incubated for 1 h with 0.1 mM labelled L-proline. Means from three experiments are shown. *R* is the ratio of experimental to theoretical accumulation.

| Preincubation | Incubation | $pH_{out}$ | $\Delta\psi$ (mV) | $\Delta$pH | pmf (−mV) | Maximum theoretical accumulation ratio | Experimental accumulation ratio | *R* |
|---|---|---|---|---|---|---|---|---|
| Aerobic | aerobic | 4.5 | −39 | 1.78 | 146 | 271 | 64 | 0.24 |
| | | 7.0 | −83 | 0.26 | 99 | 45 | 72 | 1.60 |
| Aerobic | anaerobic | 4.5 | −27 | 1.00 | 87 | 28 | 9 | 0.32 |
| | | 7.0 | −75 | 0.09 | 80 | 22 | 8 | 0.36 |
| Anaerobic | aerobic | 4.5 | −29 | 1.77 | 135 | 178 | 69 | 0.39 |
| | | 7.0 | −75 | 0.23 | 89 | 30 | 67 | 2.23 |
| Anaerobic | anaerobic | 4.5 | −24 | 1.01 | 84 | 25 | 7 | 0.28 |
| | | 7.0 | −68 | 0.09 | 74 | 17 | 6 | 0.35 |

TABLE III

INTRACELLULAR ATP LEVEL (nmol/mg DRY WEIGHT) IN *S. cerevisiae* K AFTER 1-h INCUBATION IN THE PRESENCE OF INHIBITORS AT pH 4.5

| Inhibitor added [a] | ATP | Experimental accumulation ratio | $R$ [b] |
|---|---|---|---|
| None | 1.8 | 64 | 1.00 |
| None, anaerobically | 1.7 | 7 | 0.11 |
| Diethylstilbestrol | 1.9 | 62 | 0.92 |
| Dicyclohexylcarbodiimide | 1.7 | 65 | 1.06 |
| 3-Chlorophenylhydrazono-malononitrile | 1.2 | 67 | 1.56 |
| Sodium arsenate | 0.12 | 70 | 16.2 |
| KCl | 1.8 | 62 | 0.94 |
| 2-Deoxy-D-glucose [c] | 1.7 | 66 | 1.08 |
| Antimycin [c] | 0.6 | 5 | 2.31 |
| Antimycin + 2-deoxy-D-glucose | 0.02 | 4 | 5.56 |

[a] Inhibitor concentrations as in Table I.

[b] Ratio of experimental accumulation to ATP level, normalized to 1.00 for the control.

[c] Inhibitors were added for 20 min in the presence of glucose, the suspension was then centrifuged and cells resuspended with the inhibitors for 1 h.

arsenate), while, on the other, 62 : 1 was obtained where 562 : 1 was attainable (with diethylstilbestrol at pH 4.5).

This discrepancy is difficult to explain in terms of altered pH values near the membrane due to unstirred layers [19,20] or the negative surface potential [21], as their effects should be purely kinetic in nature. A more plausible explanation appears to be the existence of a mosaic structure in the membrane which has some appeal to the experimental worker for reasons of both function and morphology [22,23]. If the $H^+$-extruding membrane system (H-ATPase in the presence of glucose, but possibly a $CO_2$ generator or a $H^+$-$K^+$ exchange reaction in its absence) is localized in the membrane in such a way that the $H^+$-driven systems are clustered around it, they may see a completely different $\Delta$pH and membrane potential than those observed in the bulk. These are the first-hand users of the electrochemical potential gradient of protons – what they do not dissipate may then appear as the bulk quantities we are apt to measure with our cell suspension-oriented probes.

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