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PROTON TRANSLOCATION DURING ANAEROBIC ENERGY PRODUCTION IN *SACCHAROMYCES CEREVISIAE*

J. C. RIEMERSMA and E. J. J. ALSBACH

Laboratory of Medical Chemistry, University of Leiden, Leiden (The Netherlands)

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SUMMARY

1. By means of a recording titrator the quantity of H^+ extruded in anaerobic energy production by *Saccharomyces cerevisiae* was measured (ΔH^+) at constant external pH. The quantity ΔH^+ corresponds to a loss of extracellular H^+ as derived from measurements of intracellular pH changes. H^+ translocation is strongly influenced by K^+ and by azide.

2. K^+ extends the linear relation between ΔH^+ and the initial glucose addition to higher glucose quantities. Various types of evidence suggest a passive role of K^+ in facilitating H^+ translocation.

3. By utilising 1-anilino-8-naphthalene sulfonate (ANS), dodecylsulfate, dodecyltrimethylammonium and Auramine O ions as probes, changes in charge distribution at the cell surface can be detected during energy production. In yeast cells such lipophilic ions do not function as membrane-permeating but as membrane-bound probes. Their uptake characteristics confirm the hypothesis of proton translocation as a primary phenomenon resulting in secondary ion movements.

INTRODUCTION

A wide range of transport systems for substrates and for inorganic ions have been found in different microorganisms. To account for the specificity of uptake processes movable "porters" in the cell membrane have been postulated. The membrane in several cases appears to function as a kind of transducer converting energy derived from metabolism into osmotic work [1]. The hypothesis has been advanced that during energy production a "porter" is chemically transformed in a phosphorylation–dephosphorylation cycle [2, 3], while the uptake of a compound or ion from the medium is connected with a resulting reversible conformation change.

It has also been suggested that energization of the cell changes membrane structure as a whole in such a way that an electrical gradient is set up across the membrane as a result of which certain ions enter the cell [4, 5]. In energized cells of *Strepto-*

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

coccus faecalis, according to Harold and co-workers [6, 7], proton translocation as a primary process establishes a trans-membrane electrical gradient responsible for the movement of K^+. This view does not exclude an essential role of "porters" in facilitating the movement of specific ions but does not require their chemical transformation.

Here experiments are reported regarding ion transport in *Saccharomyces cerevisiae* during anaerobic metabolism. In this case a primary translocation of protons may drive other forms of ion transport. As in other microorganisms, primary and secondary ion transport are hard to differentiate without direct measurements of trans-membrane potentials. Indirect evidence on this point can be obtained by comparing the characteristics of H^+ and K^+ transport, and by using certain lipophilic ions as membrane probes to detect changes in charge distribution at the outside surface of the cell.

Addition of a small quantity of glucose to an anaerobic yeast suspension causes a brief extrusion of H^+ which can be measured by a recording titrator at constant external pH. The total quantity ΔH^+ measured in this way is found to be greatly increased in the presence of K^+ beyond a certain concentration [8–10]. Apart from considering the nature of the effect of potassium the uptake and release of certain added organic ions were studied, in particular Auramine O, dodecyltrimethylammonium bromide, 1-anilino-8-naphthalene sulfonate (ANS), and dodecylsulfate.

With regard to most of these lipophilic cations and anions information is available with reference to the interaction with biological membranes as well as with lipid membrane models [11–20]. Particularly with dodecylsulfate and with dodecyltrimethylammonium ions care should be taken that no membrane damage occurs; the concentrations used should be very small [21–23]. Under the conditions of our experiments, as the results indicate, the compounds mentioned are embedded in the membrane surface rather than being bound to cytoplasmic constituents. Contrary to our expectations they did not function as membrane-permeant but as membrane-bound substances.

MATERIALS AND METHODS

Materials

The chemicals used were of reagent quality and were used without further purification: Auramine O (British Drug Houses), ANS (sodium salt) (K and K Laboratories), Azure A (Allied Chemical), Tropaeoline OO (Gurr Ltd), sodium dodecylsulfate (BDH) and dodecyltrimethylammonium bromide (gift of Unilever Research Laboratory, Vlaardingen).

Fresh baker's yeast (Koningsgist, Delft) was washed by suspension in distilled water, centrifugation, and resuspension. Prolonged aeration (16 h) was used to bring the cells in a "starved" condition. After centrifugation the packed cells were suspended in distilled water to obtain a 71% suspension (71 g fresh yeast/100 ml) which served as a stock suspension. Purified N_2 was passed through the suspension overnight to obtain complete deaeration.

Analytical methods

Recording pH-stat equipment (Radiometer) was used to follow the time course of H^+ transport and to determine the total quantity of H^+ extruded (ΔH^+) at con-

stant pH. The intracellular pH in metabolizing cells as a function of time was determined at intervals by filtering rapidly a small volume of yeast suspension over a Millipore filter (HA, 0.45 μm), and by bringing the resulting yeast cake immediately afterwards into liquid N_2. Subsequently the cakes were thawed one by one with 2 ml water and briefly heated (100 °C, 5 s); the pH of the suspension was determined after cooling (cf. ref. 24). For the purpose of determining ion concentrations in the extracellular medium as a function of time a rapid-filtration procedure was applied using Millipore filters (HA, 0.45 μm). K^+ was determined by flame photometry, and Auramine O and ANS by absorption spectrophotometry at 434 and 375 nm, respectively. Dodecylsulfate in low concentrations was determined using ^{35}S-labelled sodium dodecylsulfate. Higher concentrations were determined colorimetrically at 630 nm after extracting a complex between surfactant and Azure A into 1,2-dichloroethane (see ref. 25). Dodecyltrimethylammonium ions were determined colorimetrically at 400 nm after extracting in a similar way a complex between surfactant and Tropaeoline OO into 1,2-dichloroethane (modified from ref. 26).

In binding experiments with dodecyltrimethylammonium ions, 15 ml yeast (71%, w/v) at 30 °C and pH 5.0 was mixed with (4–*a*) ml water and *a* ml 10 mM dodecyltrimethylammonium bromide ($a = 0.5, 1, 2, 4$, respectively). After Millipore filtration dodecyltrimethylammonium was determined in the filtrate as described above. To obtain broken cells 3 ml 71% yeast was brought into 10 ml water at 100 °C, boiled for 5 s, and centrifuged after cooling. The cell cake obtained with 6 ml water was brought to 30 °C and pH 5.0; dodecyltrimethylammonium bromide (15 mM) was added (0.1, 0.2, 0.5, 1.0 ml, respectively). The surfactant ion was determined in the filtrate after Millipore filtration. Binding experiments with other substances were carried out similarly, using the analytical methods indicated.

RESULTS AND DISCUSSION

Upon addition of a small quantity of glucose to an anaerobic yeast suspension, resting cells are converted into actively metabolizing cells and rapid H^+ ejection is observed. If the extracellular pH is kept constant by a pH-stat arrangement the quantity of H^+ (ΔH^+) extruded can be determined. This quantity can be correlated with the loss of H^+ from the cytoplasm by measuring the time course of the intracellular pH as described under Materials and Methods.

As Fig. 1 shows the initially existing pH difference between external and internal pH increases during fermentation. Added K^+ enhances the resulting pH difference but also facilitates its dissipation after fermentation has ended. Azide and 2,4-dinitrophenol inhibit the rise of a trans-membrane pH gradient. In the absence of potassium or of lipophilic proton conductors the increased pH gradient is relatively stable, which indicates that normally the yeast cell membrane is a relatively effective barrier to H^+. Evidence has been presented earlier that azide and 2,4-dinitrophenol exert their action at the cell membrane rather than interfering with intracellular processes [27, 28].

A comparison can be made between (a) H^+ loss from the cell interior, and (b) H^+ increase outside the cells. The latter is registered by the titrator as the quantity of base required to keep the external pH at the set value. The internal pH change, combined with the assessed buffering capacity of the lysed cell mass, gives the quantity

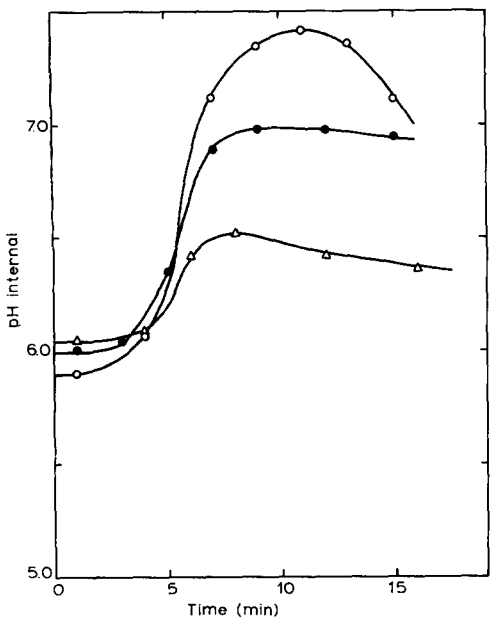


Fig. 1. Time course of intracellular pH during fermentation (for experimental conditions see Table I). (a) ●–●, no additions; (b) ○–○, KCl added (0.16 M); (c) △–△, NaN_3 added (1 mM).

of H^+ removed from the cytoplasm. Given inevitable measuring errors a reasonable agreement is found between (a) and (b); see Table I.

TABLE I

H^+ LOSS FROM CYTOPLASM COMPARED WITH H^+ APPEARING IN EXTRACELLULAR MEDIUM

Procedure: To 72 ml yeast suspension (59 %, w/v) at 30 °C and pH 5.0, maintained by recording titrator, at t = 0 min 3 ml 30 % glucose was added to start fermentation. The resulting intracellular pH changes were determined as described under Materials and Methods. From the pH change ($\Delta pH_{internal}$) and the buffering capacity the quantity of H^+ extruded was determined ($\Delta H^+_{internal}$). This value was compared with the quantity of H^+ appearing in the medium as recorded by the titrator ($\Delta H^+_{external}$).

Substance added	$\Delta pH_{internal}$	$\Delta H^+_{internal}$ (μequiv.)	$\Delta H^+_{external}$ (μequiv.)
None	1.04	1500	1550
None	1.02	1470	1490
KCl (0.16 M)	1.64	2360	2760
KCl (0.16 M)	1.42	2040	2760
NaN_3 (1 mM)	0.54	780	500
NaN_3 (1 mM)	0.41	590	600

De novo formation of acidic compounds as byproducts of fermentative metabolism apparently does not significantly contribute to the observed H^+ translocation; there is a movement of H^+ from inside to outside but no net acid production.

In resting cells the movement of H^+ into or out of the cell which can be induced by setting the external pH at a value different from an "equilibrium" value can also be measured by the recording titrator. The movements are slow, confirming the already noted effectiveness of the cell membrane as a barrier for H^+. A net H^+ movement equal to zero was found at an external value of 5.0, which is 1 pH unit lower than the internal pH (Fig. 2). On the basis of Donnan considerations, given a preponderance of negatively charged poly-electrolytes in the cell, zero net H^+ movement would be expected at a lower internal as compared to the external pH.

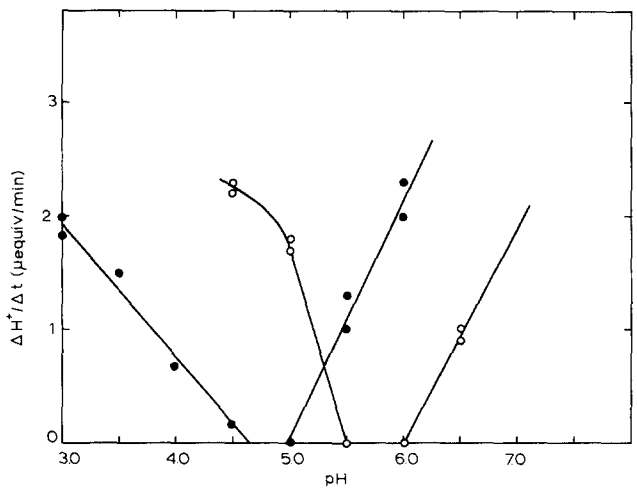


Fig. 2. Leakage of H^+ into and out of resting cells, depending on the external pH, as measured by recording titrator (Burette filled with 0.05 M Tris and 0.025 M H_2SO_4, respectively). (a) ●–●, 18 ml yeast (59 %, w/v), temperature 30 °C, no additions; (b) ○–○, idem, in the presence of 1 mM azide.

Donnan considerations cannot explain the observations reported (see also refs 29 and 30). It seems likely that asymmetric properties of the cell membrane are responsible for maintaining the asymmetric pH distribution required for zero net H^+ movement. In the presence of lipophilic-proton conductors such as NaN_3 or 2,4-dinitrophenol, which depolarize the membrane, the phenomenon is not observed and zero H^+ movement occurs when the outside and inside pH are equal (Fig. 2). Addition of such compounds not only leads to an immediate collapse of a transmembrane proton gradient but also initiates steady potassium leakage from the cells [31].

With regard to K^+ the yeast cell appears to be relatively permeable. As Fig. 1 shows the presence of 160 mM potassium facilitates the dissipation of a proton gradient established in metabolism. It has been claimed that *S. faecalis* requires valinomycin for potassium uptake during energization [7]. Yeast cells do not require a specially added potassium carrier.

Certain quantitative characteristics point to potassium transport as a secondary form of transport with proton translocation as the primary form. Thus in metabolizing yeast cells the quantity of translocated H^+ (ΔH^+) depends on the glucose quantity added in such a way that the quotient $\Delta H^+/\Delta Glc$ is maximal when ΔGlc is

close to zero. In this range of small glucose quantities potassium uptake from the medium is negligible. Significant potassium uptake begins only when more than 250 μmoles glucose is added [31]. Apparently a certain quantity of H^+ must be translocated before K^+ uptake becomes measurable; in the initial phase available intracellular anions (succinate) may accompany the extruded H^+.

Whether or not K^+ is present does not affect the optimal value of the quotient $\Delta H^+/\Delta$Glc at small glucose quantities, but potassium does extend the corresponding linear relation between ΔH^+ and ΔGlc to larger glucose quantities (Fig. 3). Normally, when the glucose quantity is increased, the curve for ΔH^+ as a function of ΔGlc levels off; H^+ extrusion appears to be self-limiting and this limitation is to some extent removed by potassium. This again points to a secondary uptake of K^+, consequent upon a primary electrogenic proton translocation.

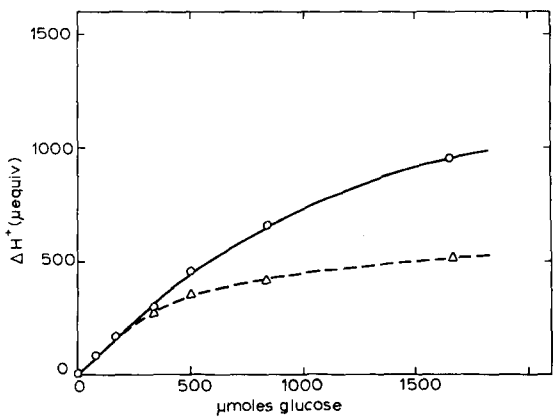


Fig. 3. Quantity of H^+ released during fermentation as a function of the glucose quantity. pH kept constant at 5.0 by titrator. To 15 ml yeast (71 %, w/v) and (3–*a*) ml water *a* ml glucose (30 %) was added. (a) ○–○, in the presence of KCl (77.5 mM); (b) △- - -△, without added KCl.

Earlier investigations led us to believe that certain organic ions would pass the membrane in response to electrical trans-membrane gradients, thus providing evidence about their direction. A positive lipophilic ion like the dodecyltrimethylammonium ion was expected to move in the same direction as K^+. In fact, as Fig. 4a shows, the movements of dodecyltrimethylammonium ions are rather complex. The measured curve was corrected for pH changes in the medium. Immediately after glucose addition there is an uptake of dodecyltrimethylammonium ions, as evident from a decrease in the concentration of this ion in the medium. The onset of energization is accompanied by a release of dodecyltrimethylammonium ions, while in a later phase (after 3 min) these ions are taken up. In this phase K^+ moves outward. A comparison of Figs 4a and 4b shows that the total pH fall is diminished in the presence of dodecyltrimethylammonium; this indicates a partial dissipation of the pH gradient (internal–external) in the presence of this ion. Similarly potassium loss after 4 min is considerably more rapid in the presence of dodecyltrimethylammonium ions.

K^+ unquestionably enters the cytoplasm. With organic lipophilic ions the possibility has to be envisaged that they do not pass the membrane but remain surface bound. Uptake changes would in that case reflect different surface-charge conditions

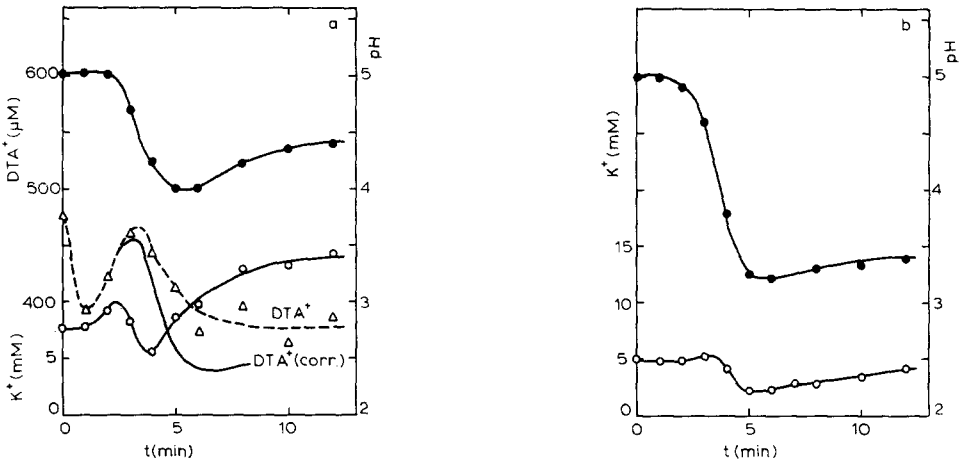


Fig. 4. Time course for pH (●–●), K^+ concentration (○–○) and dodecyltrimethylammonium (DTA^+) concentration (△- - -△) in the extracellular medium during fermentation. (a) in the presence of dodecyltrimethylammonium bromide; (b) in its absence. Conditions: 25 ml yeast (71 %, w/v)+2 ml water+3 ml dodecyltrimethylammonium bromide (10 mM), at $t = 0$ 0.5 ml glucose (30 %) added. The curve for surfactant ion concentration was corrected for the pH change of the medium.

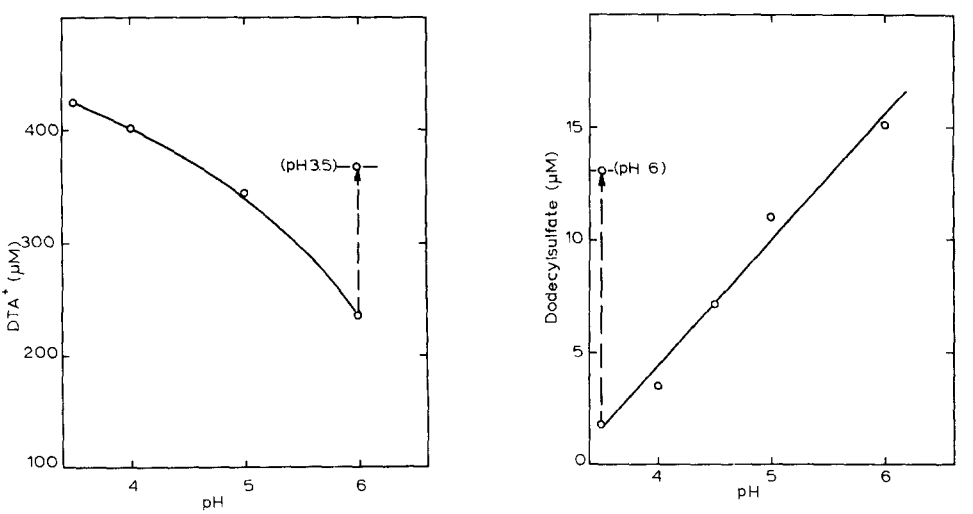
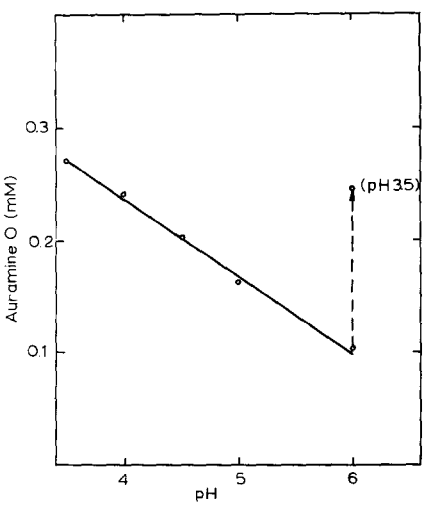


Fig. 5. Concentration of dodecyltrimethylammonium ions (DTA^+) in the medium after equilibration, as a function of extracellular pH (30 °C). Conditions: 15 ml yeast (71 %, w/v)+2 ml water +1 ml 15 mM dodecyltrimethylammonium bromide was brought to pH 3.5, 4.0, 5.0, 6.0, respectively; the final sample was subsequently brought to pH 3.5.

Fig. 6. Concentration of dodecylsulfate in the medium after equilibration as a function of extracellular pH (30 °C). Conditions: 15 ml yeast (71 %, w/v)+2 ml water+1 ml mM sodium dodecylsulfate (^{14}C labelled) was brought to pH 6.0, 5.0, 4.5, 4.0, 3.5, respectively; the final sample was subsequently brought to pH 6.0.

of the cell. Various types of experiments do in fact suggest that in resting cells ions such as dodecyltrimethylammonium, Auramine O, ANS, and dodecylsulfate are embedded in the membrane.

The extracellular pH strongly influences the extent to which all these ions are taken up by yeast cells from the medium (Figs 5–8). These differences in binding as a function of pH reflect conditions at the outer surface of the permeability barrier. As discussed above, with reference to Fig. 1, in short-run experiments the cell membrane presents an effective barrier to H^+. All the organic ions mentioned are bound reversibly, in the sense that they are removed from the cell by changing the external pH in an appropriate direction. The fact that binding is reversible supports the hypothesis of surface localization.



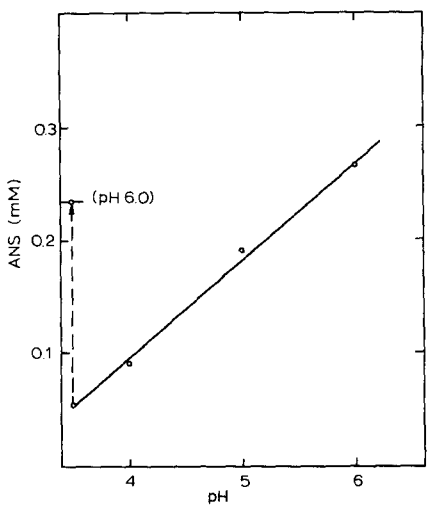


Fig. 7. Concentration of Auramine O in the medium as a function of extracellular pH. Conditions: 15 ml yeast (71 %, w/v)+2 ml water+0.5 ml Auramine O (10 mM) was brought to pH 3.5, 4.0, 5.0, 6.0, respectively; the final sample was subsequently brought to pH 3.5. Auramine O determined in filtrate after Millipore filtration.

Fig. 8. Concentration of ANS in the medium as a function of extracellular pH. Conditions: 15 ml yeast (71 %, w/v)+2 ml water+2 ml ANS (2 mM) was brought to pH 6.0, 5.0, 4.0, 3.5, respectively; the final sample was subsequently brought to pH 6.0. ANS determined in filtrate after Millipore filtration.

Removal of such ions from the cells can also be effected by other non-penetrating ions apart from H^+. Uranyl-binding sites are located at the periphery of the yeast cell, probably at the cell membrane outer surface [32]. UO_2^+ removed dodecyltrimethylammonium and Auramine O ions from the cells. Tetraphenylboron ions effectively removed dodecylsulfate (see Table II).

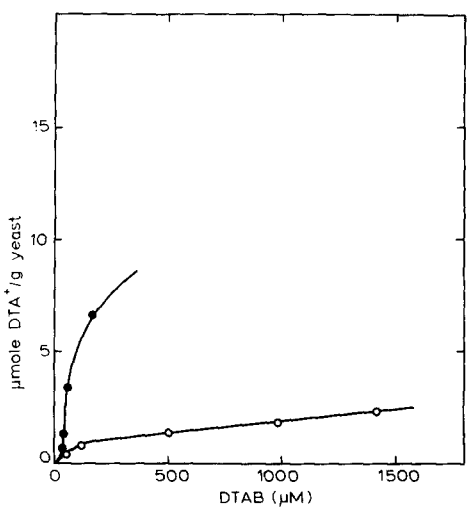
When the permeability barrier of the cells is broken, for instance by brief boiling (see Materials and Methods), the binding characteristics of the cells are found to be greatly different from those of intact cells. Broken cells bind a much greater amount of lipophilic cation (e.g. dodecyltrimethylammonium) than intact cells (Fig. 9). Binding curves for Auramine O, ANS and dodecylsulfate differed in the same way, when broken and intact cells were compared. Apparently the cell membrane masks interior binding sites and is itself mainly responsible for the binding characteristics of intact cells. The lipophilic ions considered are, on the basis of observations on resting

TABLE II

ELUTION OF LIPOPHILIC IONS BOUND BY YEAST CELLS

Procedure: To 65 ml yeast suspension (65 %, w/v) at pH 5.0 and 30 °C 5 ml 15 mM dodecyltrimethylammonium bromide was added. A sample of 2 ml suspension was removed and filtered over a Millipore filter; the surfactant cation was determined in the filtrate. The residue on the filter was washed with 5 ml 10 mM uranyl nitrate and dodecyltrimethylammonium determined in the resulting filtrate. The percentage eluted in this way was expressed as percent eluted as compared to the quantity initially bound. Procedure for other lipophilic ions: analogous, but using specific analytical methods as described under Materials and Methods.

Substance bound by yeast	Eluent	Percentage eluted
Dodecyltrimethylammonium	Uranyl nitrate (10 mM)	68.2
Auramine O	Uranyl nitrate (10 mM)	95
Dodecylsulfate	Tetraphenyl boron (sodium salt) (50 mM)	78
ANS	Tetraphenyl boron (sodium salt) (50 mM)	95



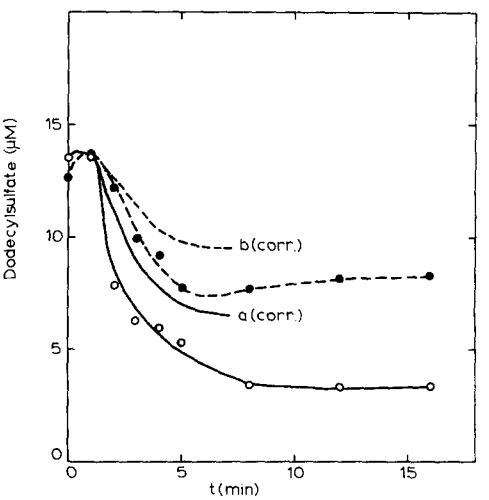


Fig. 9. Quantity of dodecyltrimethylammonium ions bound per gram yeast as a function of dodecyltrimethylammonium bromide (DTAB) concentration at equilibrium for intact cells (○–○) and cell residue (●–●).

Fig. 10. Time course for dodecylsulfate concentration in the extracellular medium during fermentation. (a) ●- - -●, in the absence of NaN_3. Conditions: 60 ml yeast (71 %, pH 5.0, 30 °C)+10 ml water+5 ml 1 mM ^{14}C-labelled sodium dodecylsulfate; at $t = 0$ 0.1 ml 30 % glucose was added. (b) ○–○, in the presence of NaN_3 (0.67 mM). The corrected curves result if pH changes in the medium are taken into account.

cells, to a large extent bound to or embedded in the plasma membrane which functions as a permeability barrier. The rapid uptake and release, followed by another period of uptake of dodecyltrimethylammonium ions in metabolising cells are likely to reflect conditions at the membrane surface (see Fig. 4a).

The course of dodecylsulfate concentration in the medium as a function of time, during fermentation, shows quite a different course. The curves, after correction

for medium pH changes, show an increased binding of dodecylsulfate ions during energization (Fig. 10). A contrast between lipophilic anions and cations emerges when ANS is compared with Auramine O. The experiments represented by Figs. 11 and 12 were done at constant pH of the extracellular medium.

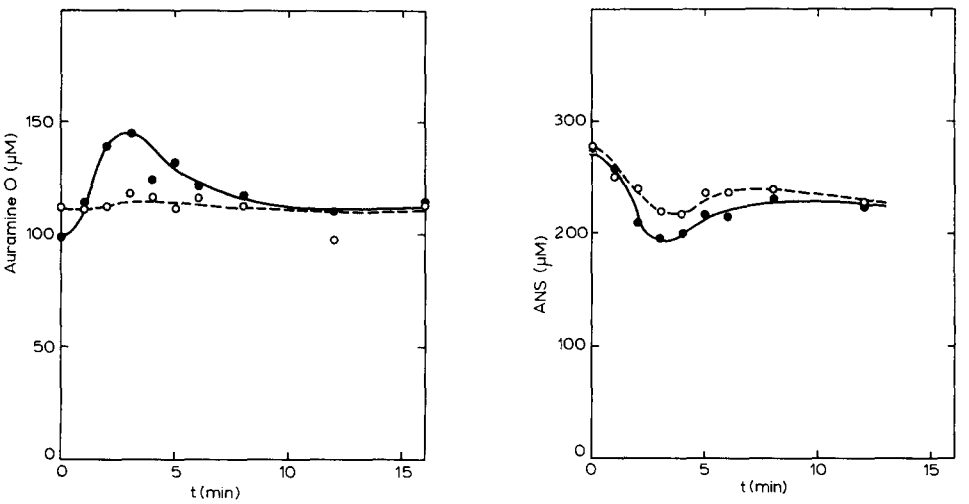


Fig. 11. Time course for Auramine O concentration in the extracellular medium during fermentation. (a) ●–●, in the absence of NaN_3. Conditions: 60 ml yeast (71 %, w/v), pH 5.0, 30 °C+13 ml water+2 ml 10 mM Auramine O; at $t = 0$ 0.2 ml 30 % glucose was added. (b) ○- - -○, in the presence of NaN_3 (0.67 mM). In both cases the pH was kept at 5.0 by a titrator.

Fig. 12. Time course for ANS in the extracellular medium during fermentation. (a) ●–●, in the absence of NaN_3. Conditions: 50 ml yeast (71 %, w/v), pH 5.0, 30 °C+5 ml water+7 ml ANS (sodium salt) (2 mM); at $t = 0$ 0.2 ml 30 % glucose was added. (b) ○- - -○, in the presence of NaN_3 (0.67 mM). In both cases the pH was kept at 5.0 by a titrator.

The concentration changes of anionic and cationic probes, as observed, support the conception of transient changes in cell surface charge as a result of H^+ translocation. In the presence of azide, which prevents the build-up of a trans-membrane proton gradient, these concentration changes are greatly diminished. It appears likely, though further experimental evidence is needed on this point, that an electrical gradient resulting from proton translocation creates the driving force for an inward movement of K^+.

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