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UPTAKE OF THE LIPOPHILIC CATION DIBENZYLDIMETHYLAMMONIUM INTO *SACCHAROMYCES CEREVISIAE*

INTERACTION WITH THE THIAMINE TRANSPORT SYSTEM

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Summary

The distribution ratio of the lipophilic cation dibenzyldimethylammonium between the cells of *Saccharomyces cerevisiae* and the medium appears to reflect changes in the membrane potential in a way that is qualitatively correct: the addition of a proton conductor or of an agent which blocks metabolism causes an apparent depolarization of the cell membrane; monovalent cations cause also a lowering of the equilibrium distribution, whereas the addition of divalent cations results in an increase of the partition ratio.

However, uptake of dibenzyldimethylammonium and probably also of other liophilic cations proceeds via the thiamine transport system of the yeast. Dibenzyldimethylammonium transport is inducible, like thiamine transport. A kinetic analysis of the mutual interaction between thiamine and dibenzyldimethylammonium uptake shows that these compounds share a common transport system; moreover, dibenzyldimethylammonium uptake is inhibited completely by thiamine disulfide, a competitive inhibitor of thiamine transport and dibenzyldimethylammonium uptake in a thiamine-transport mutant is reduced considerably.

It is concluded that one should be cautious when using lipophilic cations to measure the membrane potential of cells of *S. cerevisiae*.

Introduction

The distribution of lipophilic cations between cells and medium is often used as a measure for the membrane potential of cells that are too small to

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Abbreviation: DDA, dibenzyldimethylammonium.

permit reliable measurements with microelectrodes. For this purpose, the quaternary ammonium base dibenzyldimethylammonium (DDA) has frequently been used, e.g. in bacteria [1,2], vesicles derived from bacteria [3—5], Ehrlich ascites tumor cells [6] and mitochondria [7]. Recently we have shown that DDA distributes also across membranes of non-metabolizing cells of the yeast *Saccharomyces cerevisiae* [8].

We have examined now whether the DDA distribution can also be used as a measure for the membrane potential in *S. cerevisiae*. For another yeast, *Rhodotorula gracilis*, it has been found that quaternary phosphonium ions also distribute between the cells and the medium, and the partition ratio has been used as a measure for the membrane potential [9]. We have also applied one of these phosphonium bases, i.e., triphenylmethylphosphonium on *S. cerevisiae*.

Materials and Methods

Phosphate-starved cells of the yeast, *Saccharomyces cerevisiae* Delft 2, were aerated for one night at room temperature in distilled water in order to exhaust endogenous substrates. The yeast was centrifuged and resuspended in medium of appropriate pH at a concentration of 2.2% (w/v). The medium consisted of 45 mM Tris brought to pH 4.5 or 7.5 with succinic acid. Preincubation of the cells with substrate was carried out by adding either glucose to a final concentration of 3% (w/v) or ethanol to 1% (v/v). If not explicitely stated otherwise, preincubation was carried out with glucose anaerobically by bubbling nitrogen through the yeast suspension. Uptake of DDA, labelled with ^{14}C in the CH_3 group, was started by adding 1 vol. of cold DDA solution of appropriate concentration, containing a fixed amount of radioactive DDA, to 9 vols. of yeast suspension. Samples of 1.8 ml suspension were filtered by suction through a Schleicher and Schüll filter 602 h. A few seconds before filtration of the sample, 20 ml of 20 mM $MgCl_2$ at 0° C were added to the suction funnel. The Mg^{2+} prevented adsorption of DDA to the cell walls. On the filter the cells were washed with 2 ml of cold water. The radioactivity of the yeast cells (A_r) was determined by means of liquid scintillation counting according to [10]. Samples of 0.5 ml of the total yeast suspension (A_t) and of the supernatant (A_s) were removed at appropriate times and centrifuged. The concentration of DDA in the cell water was calculated from:

$$C_c = \frac{0.5 \cdot A_r \cdot C_0}{1.8 A_t \cdot 8.8 \cdot 10^{-3}} \quad (1)$$

The quotient 0.5/1.8 corrects for the differences in sample volume for yeast residue and supernatant and the factor $8.8 \cdot 10^{-3}$ is the ratio of the amount of cell water present in one volume of 2% yeast and one volume of the medium; the cell water amounted to 0.44 ml per g of pressed yeast [11]. C_0 is the concentration of DDA in the medium at zero time. The concentration of DDA in the medium after reaching equilibrium was $C_m = C_0 A_s / A_t$. Uptake of ^3H-labelled triphenylmethylphosphonium and of [^{14}C]thiamine was determined in a similar way, except that for thiamine uptake the final yeast concentration was 0.2% (w/v) instead of 2% (w/v) and that Eqn. 1 was adapted accordingly. Uptake of Na^+ and Rb^+ (applied as chloride salts), using $^{22}Na^+$ and $^{86}Rb^+$

as tracers, was studied according to the method described in Ref. 12. The uptake of phosphate, as Tris-phosphate, using ^{32}P as a tracer, was studied by the same method, but in this case the yeast was washed with ice-cold distilled water instead of an $MgCl_2$ solution. Initial uptake rates were determined from the slopes of the tangents to the uptake curves at zero time.

The thiamine-transport lacking mutant of *S. cerevisiae*, PT-R2 [13], was grown on Wickerhams' medium without thiamine [14] on a reciprocal shaker during 20 h at 30°C; in order to obtain phosphate-starved cells, such as the Delft II strain, the cells were transferred to medium without phosphate after 15 h of cultivation. Phosphate-rich cells of the strain Delft II were obtained by culturing the cells as described above, except that the cells were not transferred to medium without phosphate.

^{14}C-labelled DNA was synthesized according to Ref. 4. ^{3}H-labelled triphenylmethylphosphonium was a gift of Dr. W. Konings of the Laboratory of Microbiology at Groningen (The Netherlands). [^{14}C]thiamine, $^{86}Rb^+$, $^{22}Na^+$ and $^{32}P^-$ were purchased from the Radiochemical Centre, U.K. The mutant PT-R2 was kindly provided by Dr. Iwashima from the Prefectural University of Medicine at Kyoto, Japan.

Results

In order to establish whether the uptake of DDA can be used at least qualitatively as a measure for the membrane potential of *S. cerevisiae*, we have examined the effects of the 'protonconducting' uncoupler, 2,4-dinitrophenol; of the inhibitor of glycolysis, iodoacetic acid; and of several monovalent and divalent cations, upon the equilibrium distribution of DDA (Figs. 1 and 2). The cells were preincubated for 1 h in the pressure of 3% glucose, since most of our experiments on uptake kinetics are done under these conditions [12]. Both dinitrophenol and iodoacetic acid caused an efflux of DDA from cells preloaded with radioactive DDA. The effect of 1 mM dinitrophenol upon the final equilibrium distribution was the same irrespective of whether it was added together with the DDA or 30 min after the addition of DDA. Monovalent cations caused a lowering of the apparent Nernst potential (E_{DDA}), calculated from the equilibrium distribution of DDA.

$$E_{DDA} = -(RT/F) \ln(C_{cell}/C_{medium}) \quad (2)$$

R, T and F have their usual meaning and C_{cell} and C_{medium} are the concentrations of DDA in the cell water and in the medium, respectively. Possible differences in the activity coefficients between medium and cell water were neglected. Divalent cations led to more negative values of E_{DDA} if added at low concentrations. They caused a decrease towards zero again at higher concentrations.

DDA distribution was also affected by DDA itself. On increasing the external concentration of DDA above 10 μM the partition ratio decreased (Table I). Triphenylmethylphosphonium distribution showed a similar dependence on the triphenylmethylphosphonium concentration. Although 1 mM DDA decreased E_{DDA} by 84%, it had no effect on the uptake rates of Rb^+, Na^+ or phosphate (see below).

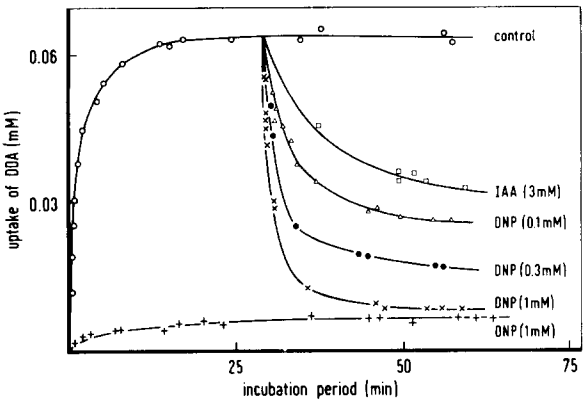

Fig. 1. Effect of 2,4-dinitrophenol and iodoacetic acid upon DDA uptake by metabolizing yeast cells. +, 1 mM dinitrophenol and 10^{-5} M DDA added simultaneously. ○, Control without additions. △, ●, X, 0.1, 0.3 and 1 mM dinitrophenol, respectively, added after 0.5 h of uptake of DDA, □, 3 mM iodoacetic acid added after 0.5 h of uptake of DDA..

If DDA crossed the cell membrane via non-mediated diffusion, simple uptake kinetics should be expected. This, however, appeared not to be the case. The kinetics of DDA uptake depended upon the time of preincubation with glucose (Fig. 3). In non-metabolizing yeast cells the rate of uptake was very low, at

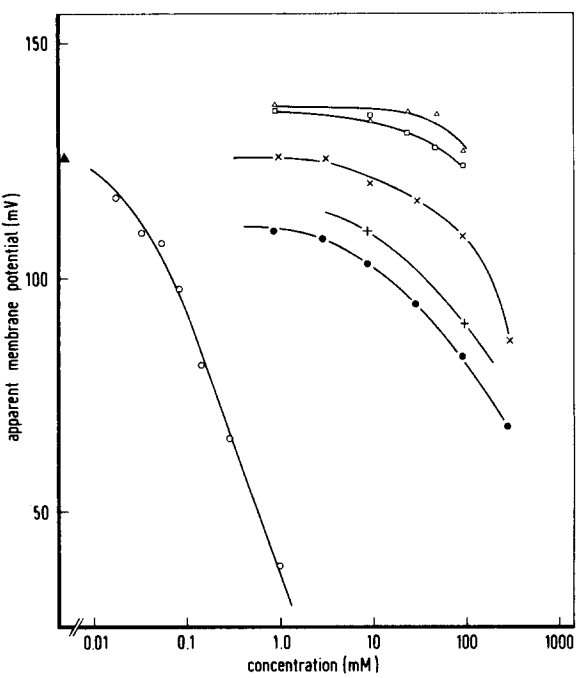

Fig. 2. Dependence of the apparent membrane potential of metabolizing yeast cells upon the concentration of various substances. The apparent membrane potential was calculated by means of Eqn. 2. The cells were preincubated for 1 h with 3% (w/v) glucose. The initial DDA concentration was 10^{-6} M. The pH was 4.5. +, Rb^+; ●, K^+; X, Na^+; □, Mg^{2+}; △, Ca^{2+}; ○, 2,4-dinitrophenol. The cations were added as chlorides.

TABLE I

DEPENDENCE OF E_{DDA} AND E_{TPMP} UPON DDA AND TRIPHENYLMETHYLPHOSPHONIUM CONCENTRATION, RESPECTIVELY

C_0 is the initial concentration in the medium. The uptake rate of 1 mM triphenylmethylphosphonium (TPMP) was too low to obtain a reliable estimate of the equilibrium distribution coefficient. Cells were preincubated during 1 h with 3% glucose; the pH was 4.5.

C_0 (μM)	$-E_{DDA}$ (mV)	$-E_{TPMP}$ (mV)
0.12	127	
1.0	127	75
10	126	56
30	110	
100	80	5.2
300	56	
1000	22	0.0

pH 4.5 and 25°C equilibrium distribution was reached only after several hours. The value of the final distribution ratio amounted to 10—22 depending on the batch of yeast [8]; this corresponded with E_{DDA} = —58 to —77 mV. The influx of DDA into cells which were preincubated with glucose under anaerobic conditions for 10 min increased considerably during the uptake. After prolonged preincubation the initial rate of uptake increased and equilibrium was established within 15 min and 8 min for 1 and 2 h of preincubation, respectively. A similar effect of preincubation was found with triphenylmethyl-

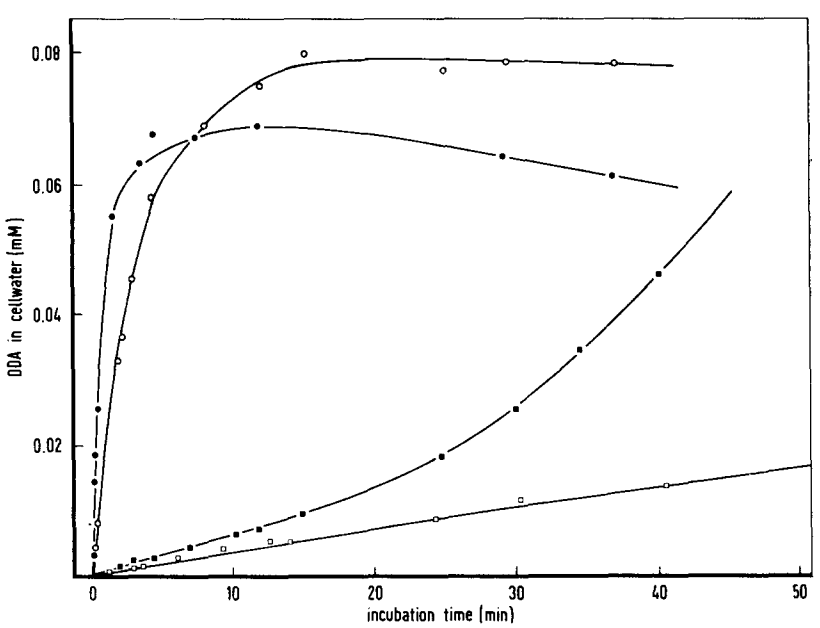

Fig. 3. The time course of the uptake of DDA after varying periods of anaerobic preincubation of yeast cells in the presence of glucose. Preincubation period in the presence of 3% glucose at pH 4.5 and 25°C: ■, 10 min; ○, 1 h; ●, 2 h; □, cells without glucose. Initial DDA concentration in medium, 10^{-6} M.

phosphonium (Table II). Contrary to the increase in the rate of DDA uptake, the equilibrium distribution decreased during prolonged preincubation with glucose; E_{DDA} fell from −140 mV to −130 mV on increasing the preincubation period from 1 h to 2 h. Similar results were obtained if the cells were preincubated under aerobic conditions with 3% glucose or 1% ethanol as a carbon source. The yeast we normally use for our experiments is phosphate-starved. Phosphate-starvation, however, had no effect on the inducibility of DDA transport; both in phosphate-starved and non-starved cells of the strain Delft II the rate of uptake after 10 min of preincubation was 2.5% of the uptake rate after 1 h of preincubation.

The initial rate of DDA uptake, determined after 1 h of preincubation with 3% glucose, increased less than proportionally with the concentration of DDA both at pH 4.5 and 7.5 (Fig. 4). The uptake rate at pH 4.5 was significantly higher than at pH 7.5 for DDA concentrations up to 30 μM. The rate of triphenylmethylphosphonium uptake also increased less than proportionally with the triphenylmethylphosphonium concentration; this rate of uptake was much lower than the rate of DDA uptake. The non-linear relation between the rate of uptake and the concentration of DDA might indicate that saturable sites were involved in the uptake of DDA into the cells. If a single-site transport mechanism were involved, a straight line should be found on plotting the rate of uptake (v) against the quotient of this rate and the concentration of DDA (v/s) according to Eqn. 3:

$$v = \frac{V \cdot s}{K_m + s} = V - K_m(v/s) \tag{3}$$

In Fig. 5 it is shown that this was not true. A concave curve as found by us might indicate the involvement of a transport mechanism, consisting of two simultaneously operating translocation processes [15], or of one translocation system with two sites [16]. In connection with the latter possibility we examined whether DDA might be translocated via the monovalent cation transport system of the yeast, which consists of a two-site translocation mechanism [17,18,19]. This cation transport system is saturated for more than 50% at a Rb^+ concentration of 3 mM, thus a competitive inhibition of DDA uptake should occur when 3 mM Rb^+ was added. Inhibition of DDA uptake by Rb^+, however, was independent of the concentration of DDA and amounted to about 60%. Moreover DDA, added at a concentration of 1 mM, did not

TABLE II

EFFECT OF THE PERIOD OF PREINCUBATION IN THE PRESENCE OF GLUCOSE UPON THE UPTAKE RATE OF TRIPHENYLMETHYLPHOSPHONIUM (TPMP) AND DDA AT pH 4.5

The rate of uptake after 1 h of preincubation is taken as 100%.

Period of preincubation	Rate of DDA uptake (%)	Rate of TPMP uptake (%)
10 min	5.5	3
1 h	100	100
3 h	150	210

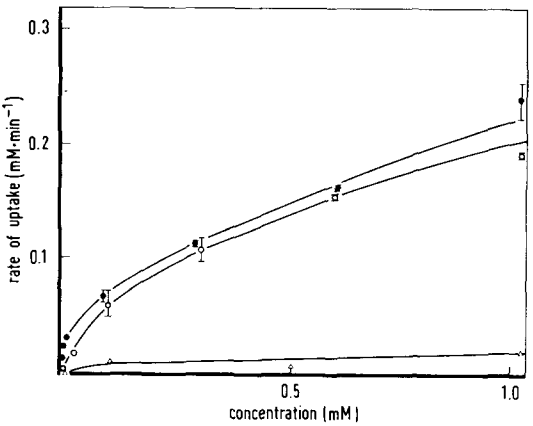

Fig. 4. Concentration dependence of the uptake rates of DDA and triphenylmethylphosphonium. •, DDA uptake at pH 4.5; ○, DDA uptake at pH 7.5; △, Triphenylmethylphosphonium uptake at pH 4.5. The cells were preincubated for 1 h in the presence of 3% glucose.

inhibit either Rb^+ or Na^+ uptake. Neither was the uptake of monovalent phosphate (which is translocated together with two or three protons [20,21] and thus as a positive complex) inhibited by 1 mM DDA (Table II).

The increase in the rate of uptake of triphenylmethylphosphonium and DDA during preincubation in the presence of glucose indicated that these cations were translocated via a physiological transport system, which was induced during the preincubation and which had an affinity to triphenylmethylphosphonium and DDA. Therefore we have looked for compounds which are known to be translocated across the yeast cell membrane and which would inhibit uptake of DDA. No inhibition was found after 1 h of preincubation of the cells in the presence of glucose, by 1 mM of either adenosine, uridine, thymidine, lysine, glycine, glutamate, glucosamine, allantoine, methylamine, ammonium, choline, acetylcholine or biotine. However, 1 mM thiamine reduced the rate of DDA uptake by 95%.

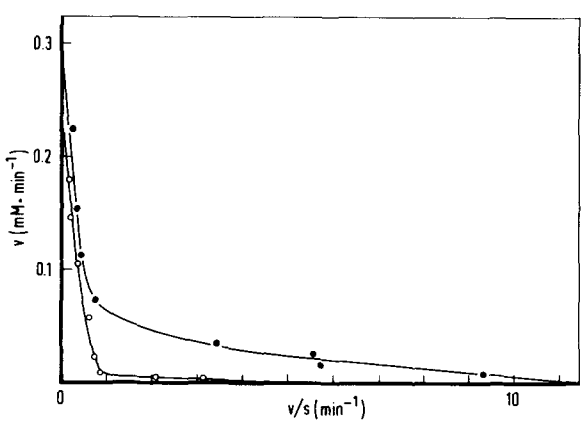

Fig. 5. Hofstee plots of DDA uptake at pH 4.5 and pH 7.5. Data of Fig. 4 are used.

TABLE III

UPTAKE RATES OF PHOSPHATE, Rb^+ AND Na^+ UNDER DIFFERENT CONDITIONS

Cells were preincubated during 1 h with 3% glucose, the pH was 4.5 unless otherwise indicated.

Yeast strain	Addition	Rate of uptake (mmol/kg dry wt. per min)		
		100 μM phosphate	1 mM Rb^+	1 mM Na^+
Delft II	none, pH 4.5	12.3	5.0	0.63
	none, pH 7.2		7.3	
	1 mM DDA	11.8	5.1	0.50
	100 μM thiamine	12.3	4.5	
	10 μM thiamine disulfide			
	pH 4.5		4.5	
	pH 7.2		9.3	
PT-R2	none	20.6	8.2	

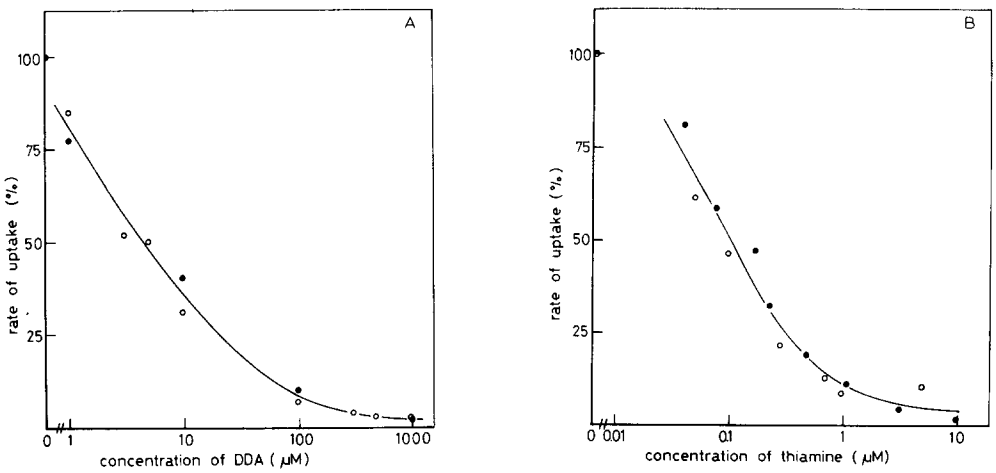

Fig. 6. The effect of unlabelled DDA (Fig. 6A) and of unlabelled thiamine (Fig. 6B) on the initial rates of uptake of labelled carrier-free thiamine (●) and of labelled carrier-free DDA (○) after 1 h of preincubation with 3% glucose at pH 4.5.

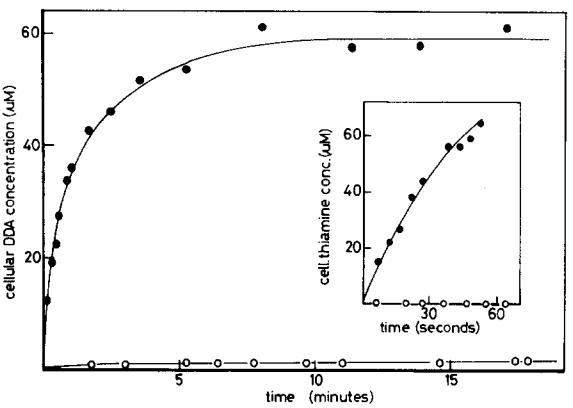

Fig. 7. Uptake of DDA by the thiamine transport mutant PT-R2 (○) and by the strain Delft II (●). Inset: uptake of thiamine by PT-R2 (○) and Delft II (●). The cells were preincubated during 1 h in the presence of 3% glucose at pH 4.5.

TABLE IV

EFFECT OF SOME LIPOPHILIC CATIONS ON THE UPTAKE OF 0.08 μM THIAMINE

The cations were added at a concentration of 100 μM, the pH was 4.5.

Cation added	Reduction in the rate of thiamine uptake (%)
Control	0
DDA	87
Triphenylmethylphosphonium	80
Tetraphenylphosphonium	70
Tetraphenylarsonium	70

Fig. 6 shows that there was a mutual inhibition by DDA and thiamine. Increasing concentrations of unlabelled DDA reduced the rate of uptake of radioactive carrier-free thiamine and of radioactive carrier-free DDA to about the same extent (Fig. 6A). Similarly, the rate of uptake of radioactive thiamine and of radioactive DDA was affected in the same way by added non-radioactive thiamine (Fig. 6B). Thiamine inhibited specifically DDA uptake: 100 μM thiamine had no effect upon the rates of uptake of Rb^+ or phosphate (Table III). DDA uptake was completely inhibited both at pH 4.5 and 7.2 by 10 μM thiamine disulfide, a competitive inhibitor of thiamine transport [22]. Finally, the uptake of DDA by the mutant PT-R2, in which thiamine transport capacity is almost completely lost [13], was strongly reduced as compared to yeast showing normal thiamine uptake (Fig. 7). The reduction in the rate of DDA and thiamine uptake in the mutant was specific; the uptake of Rb^+ and phosphate, for example, were not reduced at all (Table III).

Lipophilic cations other than DDA reduced the rate of thiamine uptake to almost the same extent as did DDA (Table IV).

Discussion

The equilibrium potential, as defined in Eqn. 2, determined from the DDA distribution at low concentrations of the cation, may reflect the membrane potential of the yeast cells in a way that is qualitatively correct. The apparent membrane potential decreases with increasing concentrations of monovalent cations, just as if found in the fungus *Neurospora crassa*, in which organism the membrane potential has been determined directly by means of inserted microelectrodes [23]. The much higher sensitivity towards K^+ than towards Na^+ is also found in this fungus. Hyperpolarization, such as we found in the presence of Ca^{2+}, is also observed with *N. crassa*; on the other hand, Mg^{2+} hyperpolarizes the cell membrane of *N. crassa* much less than does Ca^{2+}, whereas we find in *S. cerevisiae* only a slight difference between the effects of the cations. The effect of 2,4-dinitrophenol is also according to expectations: at low external pH this uncoupler is expected to cause a depolarization in view of its proton-conducting properties [24]. The decrease in the apparent membrane potential caused by iodoacetic acid may be due to its impairment of metabolism, which should lead to a decrease of a possible electrogenic membrane potential.

However, DDA does not enter the cell via simple diffusion through the cell membrane, since it is taken up via the thiamine transport system. This is concluded from the mutual interaction of DDA and thiamine uptake. Accordingly, uptake of DDA is reduced considerably by thiamine disulfide and in the thiamine transport mutant PT-R2. This explains why the rate of DDA uptake increases during preincubation of the cells with a carbon source, since the thiamine transport system is inducible [25]. Other lipophilic cations like triphenylmethylphosphonium, tetraphenylphosphonium and tetraphenylarsonium also inhibit thiamine uptake to about the same extent as does DDA (cf. Table IV). Moreover, triphenylmethylphosphonium like DDA shows an increased rate of uptake after prolonged preincubation in the presence of glucose, and a dependence on the external triphenylmethylphosphonium concentration similar to that found with DDA. The partition ratio of triphenylmethylphosphonium decreases also on increasing the triphenylmethylphosphonium concentration. Therefore it is likely that these compounds are also translocated via the thiamine transport system.

The membrane potential appears to be not the only force driving DDA uptake; on increasing the DDA concentration, the partition ratio between the cells and the medium decreases. It may be argued that this decrease is not due to a depolarizing action of the cation itself; both the uptake of Rb^+ and the uptake of phosphate remain unaffected by the presence of 1 mM DDA, a concentration at which the distribution coefficient of DDA is considerably reduced (Table II). Though not unambiguously proven, there are strong indications that Rb^+ uptake depends on the membrane potential [19,26,27]. This is also true for phosphate uptake: monovalent orthophosphate is translocated into the yeast cell with two or three protons as a positively charged complex and its uptake is assumed to be energized by the proton motive force and hence should depend on the membrane potential [20,21]. If the reduction of the accumulation of DDA cannot be ascribed to a depolarization of the cell membrane, DDA uptake must be coupled to some process which becomes limiting on increasing DDA concentrations.

If thiamine transport occurs via a mobile carrier [28], uptake of DDA will be coupled to a simultaneous efflux of counter-ions inside the cell [29], e.g. thiamine; in that case it will be determined also by factors other than the electrical potential difference across the cell membrane. Only after reaching the final equilibrium the DDA distribution may reflect the membrane potential.

Despite these objections against the use of DDA as a quantitative measure for the membrane potential, we have several indications, in addition to those described above, that the distribution coefficient of DDA reflects changes in the membrane potential in a way that is at least qualitatively correct. For example, there exists a linear correlation between the inhibition of Sr^{2+} uptake and of DDA uptake by monovalent cations and also by phosphate [30]. Butyrate, which increases DDA accumulation (data no shown), also increases the maximal rate of Rb^+ uptake [31] and phosphate decreases both the uptake of DDA and that of Rb^+ [21].

Our observations on the uptake of lipophilic cations by *S. cerevisiae* correspond in some respects with the results obtained with the uptake of quaternary phosphonium ions in the yeast *Rhodotorula gracilis* [9]. In the latter organism

a decrease of the partition ratio was also found on increasing the concentration of the phosphonium bases; and there were also differences between the distribution coefficients of the several lipophilic cations. It was not investigated, whether the uptake of the quaternary phosphonium ions by *R. gracilis* is also mediated by a physiological transport system. Orienting experiments carried out in our laboratory showed that, under the experimental conditions described in Ref. 9, the thiamine transport system is not involved in DDA translocation by this yeast species. Uptake of DDA was low and not inducible, neither was it inhibited by thiamine.

We conclude that, although changes in the distribution of DDA may reflect changes in the membrane potential of cells of *S. cerevisiae* in a way that is qualitatively correct, one should be cautious in applying the distribution coefficients of DDA and similar bases as a quantitative measure of the membrane potential.

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