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The putative electrogenic nitrate–proton symport of the yeast *Candida utilis*

Comparison with the systems absorbing glucose or lactate

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1. Strain N.C.Y.C. 193 of *Candida utilis* was grown aerobically at 30 °C with nitrate as limiting nutrient in a chemostat. 2. The washed yeast cells depleted of ATP absorbed up to 5 nmol of nitrate/mg dry wt. of yeast. At pH 4–6, extra protons and nitrate entered the yeast cells together, in a ratio of about 2:1. Charge balance was maintained by an outflow of about 1 equiv. of K^+. 3. Nitrate stimulated the uptake of about 1 proton equivalent during glycolysis or aerobic energy metabolism. 4. Studies with 3,3'-dipropylthiadicarbocyanine indicated that the proton-linked absorption of nitrate, amino acids or glucose depolarized the yeast cells. 5. Proton uptake along with lactate led neither to net expulsion of K^+ nor to membrane depolarization.

INTRODUCTION

In yeasts and related fungi the electrochemical gradient of protons acting across the plasma membrane drives the accumulation of various solutes (reviewed by Borst-Pauwels, 1981; Goffeau & Slayman, 1981; Eddy, 1982). As regards anions absorbed at pH 5, the uptake of phosphate, sulphate or glutamate is accompanied by a larger net inflow of co-substrate protons than the equivalent of the negative charges borne by the anions themselves. The resultant uptake of positive charge can be neutralized in at least three ways. (1) A spontaneous outflow of K^+ may occur. (2) Protons may be ejected through the ATPase of the plasma membrane. (3) In *Neurospora*, current injection through an inserted micro-electrode can be used to clamp the voltage (Sanders *et al.*, 1983).

Among eukaryotes, studies of nitrate uptake by algae and higher plants have shown that an accelerated proton uptake or hydroxyl-ion efflux is frequently part of the overall mechanism of nitrate assimilation. However, the role of protons both in these systems and in fungi, as co-substrates of nitrate in the above sense, is problematical (Raven & De Michelis, 1979; Deane-Drummond, 1982; Beevers & Hageman, 1983; Brownlee & Arst, 1983; Ullrich, 1983). We have found that nitrate uptake into strain N.C.Y.C. 193 of *Candida utilis* resembles phosphate uptake (Eddy *et al.*, 1980) in being greatly accelerated when the yeast is grown in a chemostat under conditions where solute availability limits the growth rate. Using ion-specific electrodes, we were then able to assay the net movements of both protons and K^+ ions that occurred during nitrate absorption. Lactate and glucose were used as control substrates in order to test the validity of the methods employed. In particular, we wished to characterize the signals emitted by a voltage-sensitive carbocyanine dye (Eddy *et al.*, 1977) in terms of the different ionic exchanges that various solutes caused. In contrast with the behaviour of other lipophilic cations, the dye signals caused by a depolarizing solute are completed in a few minutes at 25–30 °C (Van den Broek *et al.*, 1982; Hauer & Höfer, 1978).

MATERIALS AND METHODS

Yeasts

The strains of *Saccharomyces carlsbergensis* (N.C.Y.C. 74) and *Candida utilis* (N.C.Y.C. 193) were those studied previously in our laboratories (Cockburn *et al.*, 1975; Brocklehurst *et al.*, 1977). The same methods were again employed to cultivate the former yeast at 25 °C and the latter at 30 °C.

Chemostat cultures

These were 1 litre in volume in a basal mineral-salts medium and were supplied with 1 litre of air/min, as outlined in Cockburn *et al.* (1975). The cultures were kept at 30 °C, pH 4.8. (1) $(NH_4)_2SO_4$ was replaced by 1 mM- or 2 mM-KNO_3 for nitrate limitation, the modified basal medium being supplied at 0.1 l/h. (2) Glucose limitation was achieved by lowering the glucose concentration to 0.2% (w/v) in a modified basal medium supplied at 30 ml/h. In either case the outflowing yeast (400 mg dry wt.) was collected at 0 °C, washed by centrifugation (about 6×10^3 *g*-min) at the same temperature (with 3×40 ml of water) and stored for up to 4 h.

Adaptation to lactate

The glucose content of the basal mineral-salts medium was replaced by 0.25% (w/v) DL-lactate and the pH value adjusted to 5.5 by addition of KOH. The yield of yeast after aerobic growth of various batch cultures (200 ml each) at 30 °C for about 18 h varied from 0.4 to 0.9 mg dry wt./ml.

Proton absorption and K^+ efflux

These were assayed by means of a pH-electrode and a K^+-selective electrode, by using the general procedures of Brocklehurst *et al.* (1977). The latter electrode also responded to NH_4^+, which produced 30% of the response elicited by an equivalent amount of K^+ in the conditions of the assay.

(1) When nitrate was the solute to be studied, the yeast (50 mg dry wt.) was suspended in 5 mM-Tris adjusted to

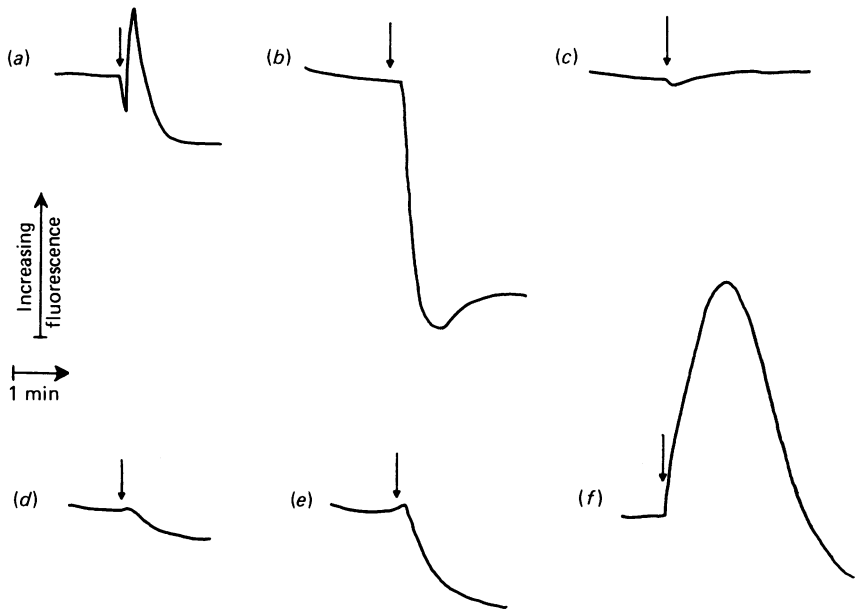

Fig. 1. Effect of glucose or maltose on the fluorescence of yeast suspensions containing either 0.3 μg or 2 μg of 3,3′-dipropylthiadicarbocyanine iodide

The yeast (*S. cerevisiae* N.C.Y.C. 74) was grown in minimal medium with either 1% (w/v) glucose (traces *a*, *b* and *c*) or 1% (w/v) maltose (traces *d*, *e* and *f*) as carbon source. The washed yeast (1 mg dry wt.) was contained in 2 ml of 2.5 mM-Tris adjusted to pH 4.8 with citric acid and containing 0.5 mM-KCl. Either 0.3 μg of the dye (traces *a* and *d*) or 2 μg (traces *b*, *c*, *e* and *f*) was added to the stirred cuvette at 30 °C. Fluorescence was excited at 630 nm and recorded at 680 nm. The fluorescence intensity having reached a steady value in about 2 min, either 25 μmol of glucose (traces *a*, *b* and *e*) or 25 μmol of maltose (traces *c*, *d* and *f*) was added at the time indicated by the arrow. Fluorescence intensity is depicted in arbitrary units as a function of time increasing from the left-hand side of each trace.

pH 4.8 with citric acid. The solution also contained appropriate metabolic inhibitors (Table 1). The detection of relatively small pH changes was facilitated by using 2.5 mM-Tris. Solutions at pH 4.2 were prepared from 5 mM-citric acid adjusted to that pH value with Tris. Solutions at pH 6 contained 5 mM-Mes adjusted to that pH value with Tris. The cell suspensions were stirred continuously at 30 °C. After the appropriate interval indicated in Table 1, the specified amount of Tris/nitrate (0.05–0.2 μmol) was added to the cell suspension. The ensuing displacements of the electrode traces (see Fig. 2) were calibrated by addition of 0.5 or 0.25 μmol of HCl or KCl.

Nitrate uptake was followed in some experiments by means of a nitrate electrode (model ISE311; EDT Research, Scientific and Medical Products, Shirley Institute, Didsbury, Manchester M20 8RX, U.K.) linked to a calomel reference electrode (type K4112; Radiometer A/S, Emdrupvej 72, Copenhagen NV, Denmark). The latter electrode was then used also as the reference for the pH electrode.

(2) The ionic movements initiated during absorption of glucose (0.1–0.25 μmol) were studied in a similar fashion. Energy metabolism of the yeast (50 mg) was inhibited by the presence of 10 mM-iodoacetamide and antimycin (10 μg). In these circumstances proton uptake accelerated for up to 0.5 min. It then returned to the basal rate. The relevant ionic displacements were therefore clearly defined.

(3) Lactate absorption at 30 °C was studied by comparing the ionic exchanges that occurred in an interval of 15 s or 30 s after the addition of 0.5 μmol of L-[U-^{14}C]lactate (2 μCi) to the yeast (50 mg). Portions (100 μl), withdrawn at intervals up to 2 min, were assayed for ^{14}C in a liquid-scintillation spectrometer. Other portions (200 μl) were mixed with water (5 ml) at 0 °C and the yeast was separated by filtration (Whatman GF/A glass-fibre filters). The ^{14}C contents of the supernatant solution and the yeast were each assayed (Brocklehurst *et al.*, 1977). Analysis of the total ^{14}C content of the cell suspension showed that it fell by only about 6% during 2 min. Accordingly the proton stoichiometry was computed by comparing the extra proton uptake during the above intervals with the corresponding ^{14}C content of the samples of filtered yeast.

Fluorescence assays

The fluorescence of solutions of 3,3′-dipropylthiadicarbocyanine (0.1–3 μg) was measured in a Perkin–Elmer luminescence spectrometer (model LS-5). The dye dissolved in 2-methoxyethanol (3–5 μl) was added to a cuvette containing yeast (1 mg dry wt.) suspended in 2 ml of 2.5 mM-Tris adjusted to pH 4.8 with citric acid. The suspension was stirred magnetically at 30 °C. Fluorescence was excited at 630 nm with a 15 nm nominal slit width, and recorded at 680 nm, as a function of time, with a nominal slit width of 10 nm. Solute absorption resulted in changes in fluorescence (Figs. 1, 3 and 4) 4–50 times larger than the fluctuation due to background 'noise'. In general a given assay was repeated with yeast grown on at least three separate occasions.

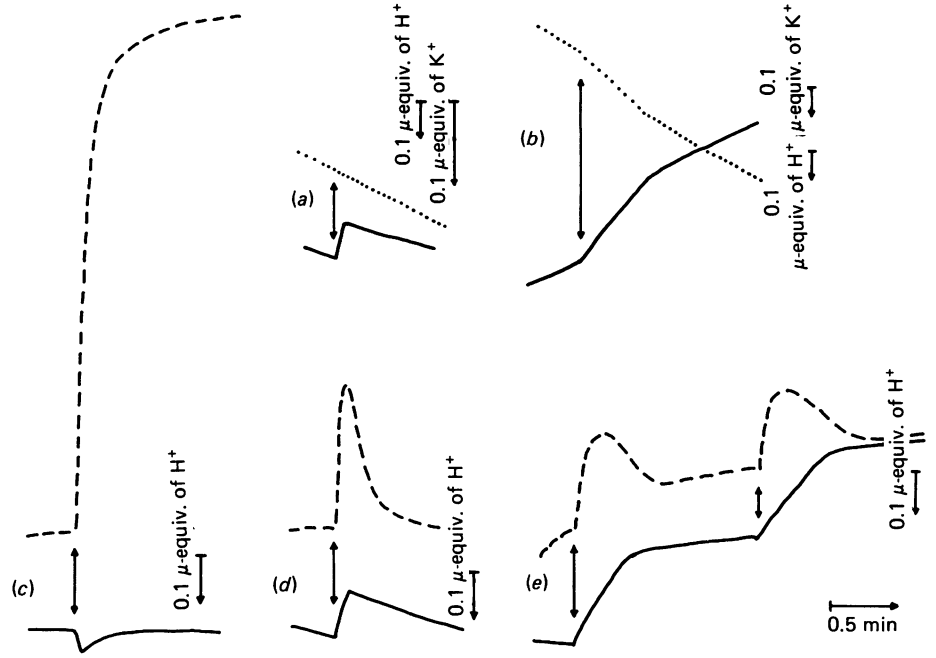

Fig. 2. Parallel recordings from the pH electrode, the K^+-selective electrode and the nitrate electrode during nitrate absorption by the preparations of *Candida utilis* grown with limiting nitrate

The yeast (50 mg dry wt. of cells) was suspended in 5 mM-Tris adjusted to pH 4.8 with citric acid and containing 0.25 mM-KCl. For the traces at (*a*), the yeast was aerated by blowing air over the stirred cell suspension, and the responses of the K^+ electrode (upper broken line) and the pH-electrode (lower continuous line) were recorded during an interval in which 0.1 μmol of Tris nitrate was added as shown. The traces at (*b*) represent similar recordings made when energy metabolism was inhibited for 5 min in the presence of antimycin (10 μg), 2-deoxyglucose (50 μmol) and iodoacetamide (25 μmol). The traces at (*c*), (*d*) and (*e*) represent recordings from the nitrate electrode (upper broken lines) and the pH-electrode (lower continuous lines). For the calibration at (*c*), the yeast was omitted and the addition of 0.1 μmol of Tris nitrate caused the signal from the nitrate electrode to increase to half its maximum value in about 7.5 s. For the traces at (*d*), 0.1 μmol of nitrate was added to the yeast (25 mg) as shown. Two such additions were made for the traces at (*e*), the yeast (50 mg) initially being put with antimycin (10 μg), deoxyglucose (50 μmol) and iodoacetamide (50 μmol) for 3 min at 30 °C. The yeast was separated by centrifugation and then suspended in 4.5 ml of 5 mM-Tris citrate at pH 4.8 containing 0.25 mM-KCl. This procedure ensured that while yeast respiration and glycolysis were inhibited, artifactual changes in the response of the nitrate electrode caused by exposure to 10 mM-iodoacetamide were minimized.

Chemicals

L-[U-^{14}C]Lactate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Antimycin, L-lactate and D-lactate were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Lactic acid used as a growth substrate was purchased from B.D.H. Chemicals, Poole, Dorset, U.K. The dye 3,3′-dipropylthiadicarbocyanine iodide was obtained from Molecular Probes Inc., 24750 Lawrence Rd., Junction City, OR 97448, U.S.A.

RESULTS

Effect of dye concentration on the fluorescence of 3,3′-dipropylthiadicarbocyanine in the presence of yeast cells absorbing maltose or glucose

Using 1 μg of the carbocyanine dye/mg dry wt. of yeast, Eddy *et al.* (1977) detected a transient increase in fluorescence after the addition of maltose to a preparation of *Saccharomyces carlsbergensis* (N.C.Y.C. 74) adapted to utilize that carbohydrate. This behaviour, which was not observed when glucose replaced maltose, was attributed to initial depolarization of the plasma membrane caused by absorption of protons through the maltose-proton symport. In contrast, Kováč & Varečka (1981) worked with about 17% of the above dose of the dye, selected so as to minimize its adverse effects on mitochondrial energy metabolism, but found no evidence of membrane depolarization with maltose. A transient depolarization observed in the presence of glucose, a carbohydrate which the yeast absorbed by facilitated diffusion, was attributed to depolarization of the mitochondrial membrane. We have now reproduced with yeast N.C.Y.C. 74 the observations made in both laboratories. Traces (*a*) and (*d*) of Fig. 1 show that, at the smaller dye concentration, glucose caused a transient depolarization, whereas maltose failed to do so. However, at the larger dye concentration, maltose caused a transient increase in fluorescence (depolarization), which was absent from the control lacking the symport mechanism (Fig. 1, traces *c* and *f*). Glucose hyperpolarized the yeast, whether it had grown with glucose as carbon source (Fig. 1, trace *b*) or with maltose (Fig. 1, trace *e*). Other work showed that the addition of maltose 2 min after glucose led to a rapid depolarization provided that the yeast utilized the former sugar (A. A. Eddy & P. G. Hopkins, unpublished work).

The initial rate of uptake of protons in the presence of maltose is a hyperbolic function of the maltose concentration and corresponded to a K_m value of 1.8 mM

Table 1. Stoichiometry of the proton absorption and K^+ efflux initiated by addition of nitrate or nitrite

Stoichiometry (±S.D. for *n* independent assays) was computed from the electrode traces in terms of the extra protons absorbed or K^+ expelled when the indicated amount of nitrate or nitrite was added to the system (see the Materials and methods section). The assays were done with 50 mg of yeast either in the presence of the named metabolic inhibitors or during glycolysis.

Assay conditions	pH	Solute added: Tris nitrate (μmol)	Solute added: $NaNO_2$ (μmol)	Stoichiometry: Equiv. of H^+ absorbed / Equiv. of NO_3^- or NO_2^-	Stoichiometry: Equiv. of K^+ ejected / Equiv. of NO_3^- or NO_2^-	*n*
After 5 min with antimycin (10 μg), 10 mM-iodoacetamide and 10 mM-2-deoxyglucose	4.8	0.05		1.96±0.33	0.90±0.24	6
		0.10		2.09±0.32	1.12±0.22	5
		0.15		2.08±0.16	0.88±0.12	5
		0.20		2.12±0.14	0.86±0.09	4
	4.2	0.10		2.08±0.13	0.71±0.32	4
	6.1	0.10		1.83±0.18	0.86±0.15	4
After 4 min with antimycin (10 μg) and 10 mM-2-deoxyglucose	4.8	0.10		1.93±0.25	0.76±0.26	11
After 5 min with 10 mM-glucose	4.6	0.10		1.10±0.08	0.02±0.14	7
After 5 min with antimycin (10 μg), 10 mM-iodoacetamide and 10 mM-2-deoxyglucose	4.8		0.10	1.76±0.30	0.70±0.26	6
After 5 min with 10 mM-glucose	4.6		0.10	1.19±0.09	Approx. 0	4

(Seaston *et al.*, 1973). In three experiments we accordingly assayed the slope of the fluorescence increase that maltose caused (Fig. 1, trace *f*) at eight different concentrations of maltose, and obtained K_m values of 0.8 mM, 1.1 mM and 1.7 mM, in fair agreement with the previous estimate based on the proton assays.

Ionic exchanges during nitrate absorption

A series of eight cultures of strain N.C.Y.C. 193 of *Candida utilis* were maintained in turn in a chemostat for periods up to 6 weeks under conditions of nitrate limitation. Both proton absorption and the efflux of K^+ induced by the addition of 0.10 μmol of Tris nitrate are illustrated in Fig. 2. During aerobic energy metabolism (Fig. 2, trace *a*), nitrate caused a fast uptake of protons of about 25 n-equiv./min per mg cellular dry wt., but no significant change in the flow of K^+. Depletion of energy metabolism, however, brought about by the joint actions of antimycin, iodoacetamide and 2-deoxyglucose, resulted in a distinct efflux of K^+ in the presence of nitrate (Fig. 2, trace *b*). The rate of induced proton absorption was then about 4–8 n-equiv./min per mg dry wt. above a basal rate of about 5 n-equiv./min per mg. Because 5 μM-nitrate caused about the same rate of proton uptake as a 50 μM solution, we conclude that the apparent K_m with respect to $[NO_3^-]$ is probably less than 1–2 μM.

Comparison of the time courses of proton uptake and nitrate absorption is made in Fig. 2, traces (*d*) and (*e*). When allowance is made for the relatively slow response of the nitrate electrode, which was half completed in about 7.5 s (Fig. 2, trace *c*), we conclude that the rate of proton movement reflected the rate of nitrate uptake in the presence of the three metabolic inhibitors. Omission of iodoacetamide resulted in a faster induced proton absorption of about 18 n-equiv./min per mg. However, a slow excretion of protons, at about 0.5–2.5 n-equiv./min per mg, then occurred in the absence of nitrate and the outflow of K^+ was almost zero. The stoichiometric relationships between the movements of K^+ and nitrate appeared to be complicated by anion excretion under these conditions.

The presence of 0.1 μmol of $NaNO_2$ accelerated proton absorption to roughly the same extent as an equivalent amount of $NaNO_3$. Addition of 5 μmol of $NaClO_3$, a putative analogue, increased proton absorption significantly, whereas 0.1 μmol was without effect.

Stoichiometry

A series of observations on the stoichiometry of nitrate and nitrite absorption are summarized in Table 1. In the presence of antimycin and deoxyglucose, either with or without iodoacetamide, about 2 equiv. of protons/mol of nitrate were absorbed, irrespective of the nitrate dose and the precise pH value prevailing. Charge neutralization was maintained largely by the efflux of K^+. In contrast, only 1 equiv. of protons was absorbed during energy metabolism maintained by glucose, and no consistent efflux of K^+ occurred. We interpret these observations as indicating that the uptake of univalent nitrate is accompanied by 2 equiv. of protons through a symport, the excess of positive charge being neutralized either by outflow of K^+ or by proton ejection through the peripheral proton pump.

Fluorescence signals with the nitrate-adapted yeast

Previous work had shown that glycine, arginine or glutamate caused an accelerated uptake of protons in preparations of strain N.C.Y.C. 193 of *Candida utilis*

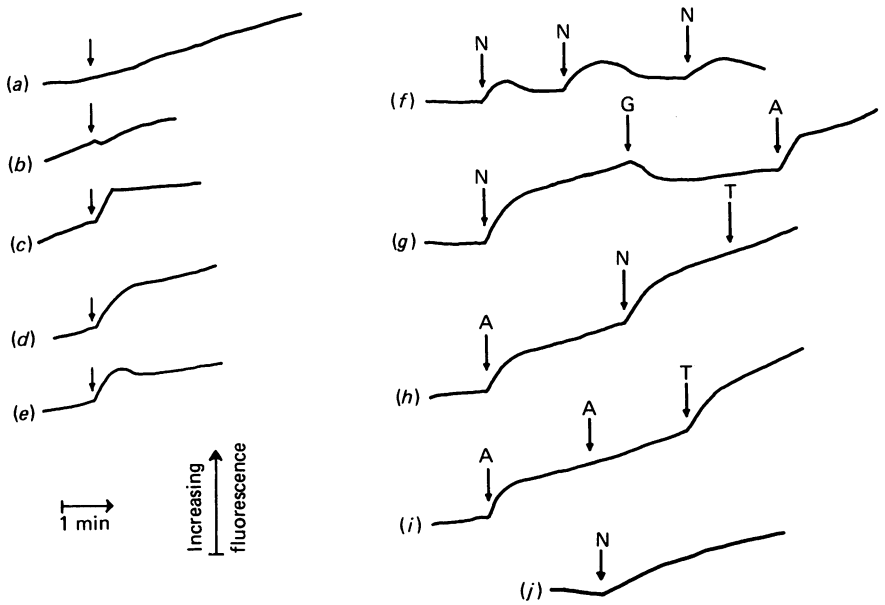

Fig. 3. Fluorescence responses exhibited with diverse substrates by nitrate-adapted *Candida utilis* in the presence of 3 μg of 3,3′-dipropylthiadicarbocyanine iodide

The assays, each using 1 mg of yeast suspended in 2 ml of Tris/citrate buffer at pH 4.8, followed the procedure outlined in the legend to Fig. 1. For trace (*a*), which served as one control, 200 μl of water was added at the arrow. Trace (*b*) is a further control in which 200 μl of water containing 200 μmol of NaCl was added. Trace (*c*) refers to the addition of 200 μmol of KCl, which was expected to depolarize the yeast. Traces (*d*) and (*e*) illustrate the consequence of adding a solution (10 μl) representing 100 nmol of glycine or glutamate respectively. For trace (*f*), 2 nmol, 4 nmol and 2 nmol of Tris nitrate were added in turn at N. For trace (*g*), 100 nmol of Tris nitrate was added at N, then 10 μmol of glucose at G and 100 nmol of L-arginine at A. For trace (*h*), successive additions comprised 100 nmol of arginine at A, 100 nmol of nitrate at N and 100 nmol of $NaNO_2$ at T. For trace (*i*), 100 nmol of L-arginine at A was followed by a further 100 nmol and then 100 nmol of $NaNO_2$ at T. For trace (*j*), the yeast cell suspension contained 10 μmol of glucose, and 100 nmol of nitrate was added at N.

grown with glutamate as the exclusive source of nitrogen (Eddy *et al.*, 1977). Preliminary observations showed that similar phenomena occurred in the washed yeast cells grown with limiting nitrate. These preparations also gave clear evidence of fluorescence changes, equivalent to depolarization, after the addition of KCl, glycine, L-glutamate or L-arginine (Fig. 3, traces *c*, *d*, *e* and *h*). Glucose caused hyperpolarization (Fig. 3, trace *g*). Furthermore, relatively small amounts of nitrate (2–10 nmol) caused a significant increase in fluorescence (Fig. 3, trace *f*), the transient nature of the phenomenon presumably reflecting the progressive absorption of the anion. The presence of 0.1 μmol of nitrite also depolarized the yeast, addition of nitrate beforehand abolishing the effect (Fig. 3, traces *h* and *i*). Although nitrate and nitrite thus appeared to share the same carrier, the observations indicated that the effects of nitrate and of arginine were independent (Fig. 3, traces *g*, *h* and *i*). In various other assays, as illustrated in trace (*j*) of Fig. 3, the addition of nitrate after glucose led to depolarization.

Symports for lactate and glucose expressed in yeast grown with lactate

A further opportunity to test the above interpretation of the fluorescence signals is provided by the circumstance that yeast N.C.Y.C. 193 grown with lactate as carbon source then absorbed glucose with protons, a roughly equivalent number of K^+ ions being expelled (Fig. 4, trace *e*). In contrast, L-lactate uptake, as illustrated in traces (*f*), (*g*) and (*h*) of Fig. 4, was associated with an accelerated uptake of protons, but no net outflow of K^+. D-Lactate evoked a similar response (P. G. Hopkins & A. A. Eddy, unpublished work). By analogy with the behaviour of the maltose–proton symport, we therefore expected that glucose, but not lactate, would cause an increase in fluorescence in the presence of the carbocyanine dye. The observations shown in traces (*a*), (*b*) and *c*) of Fig. 4 are consistent with that view.

Co-substrate stoichiometry

By using the procedures described in the Materials and methods section, the amount of [^{14}C]lactate absorbed in 15 s or 30 s was compared with the number of extra protons entering the yeast cells during the same interval. The ratio (mean ± S.E.M.) of these two quantities was 1.16 ± 0.12 (3) at 15 s and 0.86 ± 0.19 (4) at 30 s. Analogous assays performed with glucose led to a ratio of 1.14 ± 0.06 (7) extra proton equiv./mol of glucose absorbed, the accompanying efflux of K^+ being 1.18 ± 0.07 (7) equiv./mol.

We wish to stress that the accelerated uptake of protons in the presence of glucose was not observed in the yeast preparations, like those utilized for Figs. 2 and 3, which were grown with 1% (w/v) glucose as the exclusive source of carbon. However, three independent cultures of strain N.C.Y.C. 193 grown in the chemostat under conditions of glucose limitation (see the Materials and methods section) exhibited an accelerated proton absorption on glucose addition (cf. Fig. 4, trace *e*).

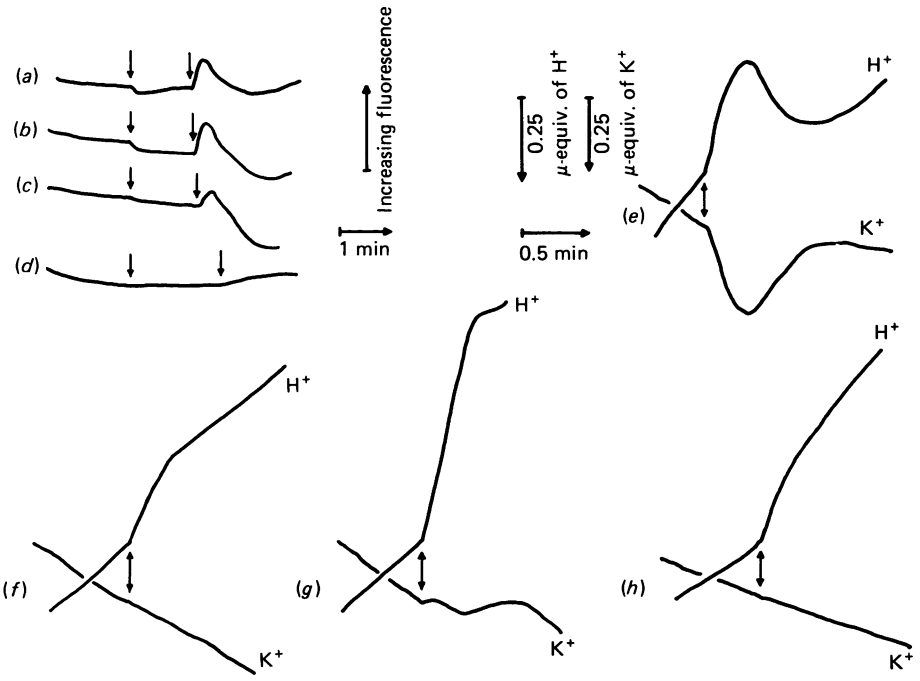

Fig. 4. Comparative effects of glucose or L-lactate on the movement of protons or K^+ and dye fluorescence

Candida utilis (N.C.Y.C. 193) was grown with DL-lactate as carbon source (see the Materials and methods section). Stirred suspensions of the washed yeast cells (50 mg) were kept at 30 °C in 5 mM-Tris, adjusted to pH 4.8 with citric acid and containing 0.5 mM-KCl. A stream of air (traces *e*, *f* and *g*) or nitrogen (traces *h*) was maintained over the surface of the suspension. The following solutes were added as shown. For traces (*e*), 0.5 μmol of glucose was added; for traces (*f*), 0.12 μmol of lithium L-lactate; for traces (*g*) and (*h*), 0.5 μmol of lithium L-lactate. Traces (*e*–*h*) for H^+ and K^+, respectively, refer to the same scale as shown. The fluorescence assays followed the procedures outlined in the legend to Fig. 1. Traces (*a*), (*b*), (*c*) and (*d*) involved, respectively, 0.5, 2, 3 and 6 μg of 3,3′-dipropylthiadicarbocyanine iodide. In each case 1 mg of yeast was contained in 2 ml of 2.5 mM-Tris adjusted to pH 4.8 with citric acid. Then 0.5 μmol of L-lactate and 10 μmol of glucose were added in turn at the indicated times, progressing from the left-hand side of the trace.

DISCUSSION

Interpretation of the effects of nitrate on the fluorescent dye response

The above observations extend previous indications (Eddy *et al.*, 1977; Van den Broek *et al.*, 1982) that, during energy metabolism, symport of protons with fermentable or non-fermentable carbohydrates produces a fluorescence increase in the presence of 3,3′-dipropylthiadicarbocyanine that reaches a maximum in 1–2 min. The above behaviour contrasts with the relatively slow response of other lipophilic cations, such as tetraphenylphosphonium, to changes in the yeast membrane potential (Hauer & Höfer, 1978; Van den Broek *et al.*, 1982; Boxman *et al.*, 1984; Eraso *et al.*, 1984).

Kováč & Varečka (1981) failed to observe a membrane depolarization in the presence of maltose apparently because they used a sub-optimal dye concentration (Fig. 1). The probe may then be absorbed mainly into the mitochondria and produce signals reflecting the membrane potential of that organelle rather than the plasma membrane (Kováč & Varečka, 1981).

The present work extends in the following ways the circumstances in which the dye response was correlated with a putative membrane depolarization. (1) Preparations of *Candida utilis*, depleted of ATP, absorbed extra protons with glucose and released K^+ ions, all in roughly equivalent amounts. Addition of glucose, to an aerobic yeast suspension containing the dye, caused an initial increase in fluorescence corresponding to membrane depolarization (Fig. 4). Subsequently membrane repolarization occurred (Fig. 4). In contrast, yeast preparations lacking the glucose–proton symport only exhibited the hyperpolarization phase (Fig. 3). (2) Lactate uptake, which was accompanied by a roughly equivalent amount of extra protons but no accelerated outflow of K^+, appeared to be an electroneutral process. Indeed, lactate failed to depolarize the yeast (Fig. 4). (3) The operation of the amino acid symports (Eddy *et al.*, 1980) for glycine, L-glutamate or L-arginine resulted in a significant increase in dye fluorescence, an effect mimicked by the addition of K^+ to the cell suspension (Fig. 3). Thus, by analogy with these diverse responses, we suggest that the effects of nitrate absorption on the fluorescence signal mean that nitrate uptake during energy metabolism also tended to depolarize the plasma membrane (Fig. 3).

Proton uptake during nitrate absorption

When proton ejection was hindered by depletion of ATP in the presence of antimycin, deoxyglucose and iodoacetamide (Kováč & Varečka, 1981; Eddy, 1982; Boxman *et al.*, 1984), the yeast preparations absorbed up to about 5 nmol of nitrate/mg of cellular dry wt. in 1–2 min. About 2 equiv. of extra protons per nitrate equivalent entered the yeast cells and 1 equiv. of K^+ left them (Table 1). Our previous studies of the phosphate and

glutamate symports have shown that it is under these conditions of metabolic depletion that the intrinsic stoichiometry of the proton symport, uncomplicated by the working of the peripheral proton pump, is likely to be exhibited (Eddy, 1978, 1982). Indeed, during glycolysis, the apparent proton stoichiometry with nitrate was about 1 (Table 1). We interpret this behaviour in terms of the rapid recycling of 1 equiv. of the 2 equiv. of protons absorbed along with nitrate. Clearly the ejection of a further 1 equiv. of protons requires, for instance, the simultaneous uptake of a cation such as K^+. This was evident during phosphate absorption (Eddy *et al.*, 1980) and glutamate absorption (Eddy, 1980), but failed to occur with nitrate (Fig. 2). The fluorescence response corresponding to a diminution of the negative membrane potential, which was observed during nitrate uptake in the presence of glucose (Fig. 3), is consistent with the above hypothesis, namely, that the intrinsic stoichiometry of the proton symport corresponds to a constant $2H^+$ per NO_3^-. The readiness with which that proton flow is masked during energy metabolism in our yeast preparations raises the question whether the analogous pH changes observed in algal and plant systems are complicated by proton recycling (Deane-Drummond, 1982; Ullrich & Novacky, 1981; Ullrich, 1983).

Ullrich (1983) has suggested the different possibility that the apparent stoichiometry of proton absorption with nitrate might increase by up to 1 equiv. of protons if nitrate were reduced to NH_3 intracellularly and the latter were expelled into the surrounding medium. This interpretation is unlikely to apply to our observations, for the following reason. The selectivity of the K^+ electrode that we used is such (see the Materials and methods section) that an improbable 3 equiv. of NH_4^+ would be required to produce the response observed during uptake of 1 equiv. of nitrate (Fig. 2, trace *b*). We are now seeking yeasts with a genetic lesion affecting the thermostability of nitrate reductase in an attempt to characterize further the properties of the proposed nitrate–proton symport.

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