BBA 76084

THE MECHANISM OF INHIBITION OF ANAEROBIC PHOSPHATE UPTAKE BY FATTY ACIDS IN YEAST

G. W F H BORST-PAUWELS AND J DOBBELMANN
Laboratory of Chemical Cytology, University of Nijmegen, Nijmegen (The Netherlands)
(Received June 26th, 1972)

SUMMARY

1. It has been shown that inhibition of anaerobic phosphate uptake in yeast by short-chain fatty acids is determined mainly by the degree of acidification of the cells caused by accumulation of these acids into the cells.

2. There is no correlation between the concentration of the fatty acid anions inside the cell and the extent of inhibition.

INTRODUCTION

Fatty acids are capable of inhibiting anaerobic phosphate uptake in yeast as shown by Samson *et al.*[1]. This inhibition cannot be ascribed to a decrease in the rate of glycolysis, because fermentation is much less sensitive to fatty acids than phosphate uptake. Borst-Pauwels and Jager[2] showed also that the concentration of ATP was not much affected by the fatty acids, and that the site of inhibition was the transport mechanism and not the metabolic fate of phosphate.

The concentration of the acid in the medium needed for a certain degree of inhibition of phosphate uptake decreases with increasing chain length of the acids[1]. We wondered whether this also holds for the concentrations of the inhibitory acids inside the cell, since the inhibition occurs only after accumulation of the acids into the cell[3]. A lack of specificity of the inhibitory action of the acids may indicate that the inhibition is not due to an interaction with proteins as suggested by Samson *et al.*[1] but to an indirect effect, which may be acidification of the cells. A decrease in cell pH on adding fatty acids to yeast has been observed by Neal *et al.*[4] and by Suomalainen and Oura[5].

METHODS

The yeast (*Saccharomyces cerevisiae* Delft II) is preincubated anaerobically at a concentration of 1 or 2.2 % (w/v) for 1 h at pH 4.5 and 25 °C in a 0.1 M sodium citrate buffer provided with 3 % glucose and 10 mM KCl. Rates of phosphate uptake were determined with 0.1 mM potassium phosphate labelled with ^{32}P according to Borst-Pauwels and Jager[2] at a yeast concentration of 0.5 % (w/v). Uptake of 1-^{14}C labelled acids was determined by centrifuging the yeast suspension at appropriate times and by subsequent assay of 0.5 ml of the supernatant (A_{sup}) for radioactivity

by means of liquid scintillation[6]. We also determined the radioactivity of 0.5 ml of the suspension (A_{sus}). The concentration of the acids inside the cell (C_i) is given by

$$C_i = [A_{sus}(V_e + V_i)V_e^{-1} - A_{sup}]V_e \cdot V_i^{-1} \cdot SA^{-1}$$

and the concentration of the acid in the medium equals $C_e = A_{sup}SA^{-1}$. In these equations SA is the specific activity, V_e and V_i are the volumes of the medium and the cell water, respectively. The latter volume amounts to 0.44 ml per g of pressed yeast[7]. The uptake of acetic and formic acid is determined by filtering 0.8 ml of yeast suspension on Schleicher and Schüll No. 602h filters, washing rapidly with 1 ml of ice-cold water and transferring the filter paper supporting the yeast to 10 ml of the scintillation liquid[6] together with 0.5 ml of distilled water and a few drops of 1 M $HClO_4$.

Cell pH values are determined according to Borst-Pauwels and Dobbelmann[7] by filtering 4 times 10-ml samples of 2% yeast and by washing the cells rapidly with 2 ml of ice-cold water, whereafter the yeast is preserved in liquid N_2. The pH is determined with a glass electrode after adding 0.5 ml of water to the pooled four samples and boiling the yeast for 30 s.

Losses of fatty acid from the yeast cell during the washing in this procedure were determined by incubating the yeast for 5 min with labelled fatty acids and filtering these cells. The radioactivity of the cells before and after washing with 2 ml of ice-cold water is compared. Corrections for adhering medium are made by adding 0.1 mM [^{14}C]-labelled mannitol in a separate experiment to the yeast just before filtration.

We have also used an indirect method for the determination of the cell acidification which is rapid enough to give us information about relative changes in cell pH after only 1 min incubation with the acidic inhibitors and in which no losses of the acid can occur. This indirect method is based upon the fact that fatty acids penetrate the yeast cell only in their undissociated form[8]. Then the distribution ratio of the acid between cells and medium is determined by the cell pH, provided that equilibrium between cells and medium is established and that the acid is not metabolized by the cells[9]. Existing methods based upon this principle appeared not to be applicable. 2,4-Dinitrophenol used by Neal *et al.*[4] penetrates the yeast cell also as anion[10] and bromophenol blue used by Kotyk[11] is not absorbed by our cells[7].

It appeared that caproic acid might be suitable for our purpose. Caproic acid penetrates into the yeast cell very rapidly. Accumulation equilibrium is reached within 1 min. The uptake is almost completely reversible, indicating that metabolic conversion of caproic acid in the cells does not contribute much to the caproate distribution between cells and medium.

Distribution of labeled compounds ($1 \cdot 10^{-6}$ M ^{14}C-labelled caproic acid and about 1 mM ^{36}Cl as NaCl) between boiled cells, or between cells made permeable to small molecules by 10 times repeatedly freezing and thawing[7], and medium is studied by adding the appropriate labelled compound to 5 ml of a 2% (w/v) suspension of boiled cells in 45 mM Tris–HCl of pH 6.3–7.0. The yeast is centrifuged after 1 min and the radioactivity of both 0.5 ml of the supernatant and of the residue is determined by means of liquid scintillation. The water content of the cells

is determined by drying the residue at 105 °C overnight. The ratio of bound and free caproate (the concentration of the undissociated acid is negligible at the pH values applied) in the cells (f_{ads}) is calculated as follows. The distribution ratio of [^{14}C]caproic acid between boiled cells or frozen and thawed cells and the medium (r_{Co}) is divided by the distribution coefficient for chloride (r_{Cl}) in order to account for the contribution of the Donnan potential between cells and medium. f_{ads} is given by Eqn 1. The factor 1.15 is the quotient of the water content of boiled cells and the cell water content of whole cells.

$$f_{ads} = (r_{Co} \cdot r_{Cl}^{-1} - 1)1.15 \quad (1)$$

RESULTS

The yeast is preincubated for 1 or for 5 min with the acidic inhibitor involved and the initial rate of radioactive phosphate uptake is then determined, the internal acid concentration and the cell pH are also determined. This cell pH could only be determined after the 5-min preincubation since the time needed for filtering the yeast cell suspension exceeded already the 1-min preincubation period. A second disadvantage of the direct method is that part of the acids accumulated are washed out again during washing the cells with 2 ml of ice-cold water. The losses of caprylic acid amount to about 30 % and those of caproic acid are irregular and vary from 0–30 %. This would mean that only cell pH values determined with the lower, more slowly penetrating[8] fatty acids (formic, acetic, propionic and butyric acids) will be reliable. Therefore, we applied the indirect method for measuring cell acidification, namely, the method based upon changes in caproate distribution ratio with changes in cell pH. The small amounts of caproate added to the yeast suspension appeared to affect neither the cell pH nor the rate of anaerobic phosphate uptake. Theoretically the distribution of caproic acid between cells and medium will be given by Eqn 2.

$$f = \frac{C_{t,i}}{C_{t,e}} = \frac{(C_{HA,i} + C_{A,i})(1 + f_{ads})}{C_{HA,e} + C_{A,e}} = \frac{(1 + K' a_{H,i}^{-1})(1 + f_{ads})}{1 + K' a_{H,e}^{-1}} \quad (2)$$

C_t is the concentration of the total acid in the medium (e) or in the cell water (i). C_{HA} and C_A refer to the free species concentrations of the acid (HA) and the anion (A^-), respectively. a is the proton activity and $K' = 10^{-4.8}$ is the apparent dissociation constant of caproic acid. f_{ads} is the ratio of bound and free caproate in the cells.

With $r_{Co} = 1.04$ and $r_{Cl} = 0.75$ (see Eqn 1 under Methods) f_{ads} equals 0.45 with an S.E. of 0.09 (6 determinations) at pH 7.0. This value increased when the pH was lowered. It appeared that between pH 7.0 and 6.3 the following relation holds as an approximation:

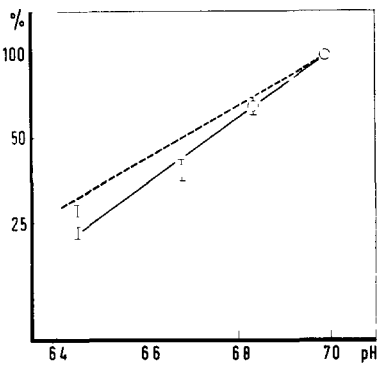
$$\log (1 + f_{ads}) = 0.161 + 0.055\,(7.0 - pH_i) \quad (3)$$

The adsorption of caproate appeared to be hardly affected by the amounts of fatty acids we are dealing with in our inhibition studies. There were no significant differences between f_{ads} values found with boiled yeast cells or with 10 times frozen and thawed cells, which shows that boiling does not much affect the adsorption capacity.

of the yeast macromolecules. After elimination of $(1 + f_{ads})$ in Eqn 2 by making use of Eqn 3 and taking $1 + K'a_{H,i}^{-1} = K'a_{H,i}$, which is allowed when the cell pH is much greater than log K', Eqn 2 can be transformed as follows:

$$\log f = \log K' + 0.161 + 0.055 \times 7.0 - \log (1 + K'a_{H,e}^{-1}) + 0.945\ pH_i^{\dagger} \qquad (4)$$

This means that log f will be proportional to the cell pH (pH_i). Fig. 1 shows that the observed decrease in f is only slightly greater than the decrease in f expected theoretically according to Eqn 4. The mean value of f found with non-inhibited cells (139 ± 5, 27 determinations) did not differ significantly from the value of f calculated by using the values of pH_i obtained with the glass electrode in the boiled cell suspension. This pH was 6.97 ± 0.02 (14 determinations) and corresponds with an f value of 143 ± 6.



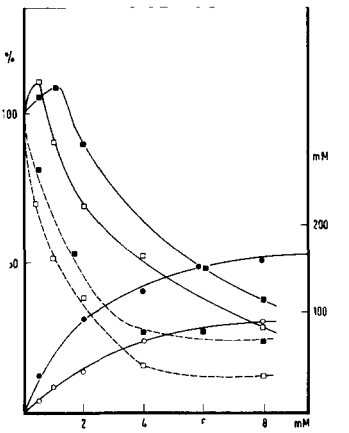


Fig 1 Dependence of the relative caproic acid distribution ratio (f) expressed in per cent of the control upon the cell pH, determined with the glass electrode - - - -, line expected according to Eqn 1 The cell pH values are varied by incubating the yeast with varying amounts of formic acid, acetic acid, propionic acid and butyric acid The height of the bars is a measure for the S E 100% caproic acid corresponds with a distribution ratio amounting to 139

Fig 2 The effect of varying butyrate concentrations upon the rate of anaerobic phosphate uptake, the distribution ratio of caproic acid between cells and medium and the internal butyrate concentration Open symbols refer to 1-min preincubation, closed symbols refer, to 5-min preincubation, □, ■. phosphate uptake (——) and caproic acid distribution (- - - - -) Both values are expressed in per cent of the control ○, ● internal fatty acid concentration in mM The scale of these concentrations is given at the right side of the figure The rate of phosphate uptake of the control is 15 mmoles·min^{-1}·kg^{-1} dry weight of the yeast

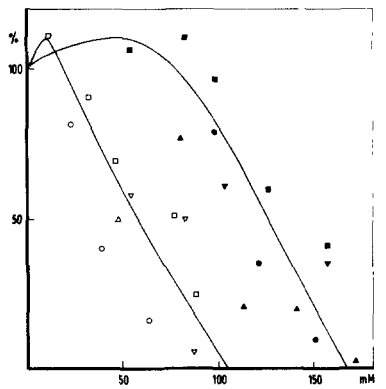
Fig. 2 shows typical results of the phosphate uptake studies. Though we measured the unidirectional influx rather than the net uptake of phosphate our data may probably also apply to net phosphate transport, since exchange diffusion is of minor importance in yeast[12]. It appeared that inhibition of phosphate uptake by butyric acid is accompanied by a decrease in the caproate distribution ratio.

Inhibition of anaerobic phosphate uptake is more effective after only 1 min preincubation with butyric acid than after 5 min preincubation, in spite of the fact that the internal butyric acid concentration is considerably higher in the latter case Thus the sensitivity of the yeast to the butyrate in the cells decreases on

extending the time of preincubation from 1 min to 5 min. Similar results are obtained with formic acid, acetic acid and propionic acid.

Fig 3 shows a plot of the phosphate uptake rate against the internal fatty acid concentration at both times of preincubation with these acids. The values for butyric acid are also included. The decrease in the rate of phosphate uptake is much greater at only 1 min preincubation than at 5 min preincubation, when comparing the values obtained at a certain fatty acid concentration in the cells. This is also the case with yeast treated with caproic acid, although the shift in sensitivity of the yeast to the acid in the cells is then less than observed with the other acids, see Fig 4. No change in sensitivity of the yeast to caprylate is observed.

It is seen from Fig. 2 that the acidification of the cells, as measured *via* the



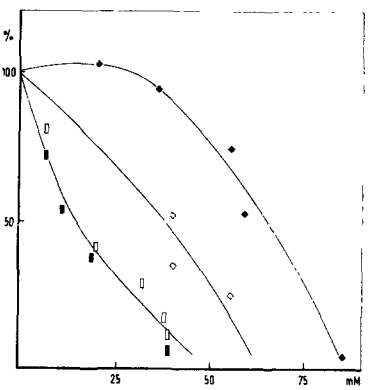


Fig 3. Plot of the rate of phosphate uptake against the concentration of the acids in the cell. The concentrations of the acids applied varied as follows: 0–80 mM formic acid (○), 0–60 mM acetic acid (△); 0–25 mM propionic acid (▽); 0–8 mM butyric acid (□) See also subscript to Fig 2

Fig 4 Plot of the rate of phosphate uptake against the concentration of the acids in the cell. The concentrations of the acids applied varied as follows· 0–5 mM caproic acid (◇); 0–1 mM caprylic acid (□). See also subscript to Fig. 2

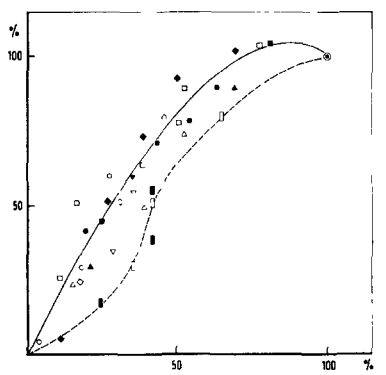


Fig 5. Dependence of the phosphate uptake rate upon the distribution ratio of caproic acid. - - - -, drawn through the points obtained with caprylic acid; ——, refers to the data obtained with the other acids See Figs 3 and 4 for the meaning of the symbols.

distribution ratio of caproic acid is less at any butyrate concentration in the medium after the 5-min preincubation than after the 1-min preincubation. There is a parallel between the changes in caproate distribution and the changes in phosphate uptake rate, apart from the small significant increase in the rate of phosphate uptake observed at the lower butyrate concentrations. A clear relationship is shown by plotting the rate of anaerobic phosphate uptake *versus* the relative value for the caproate distribution, see Fig. 5.

The data obtained after 1 min preincubation and those after the 5-min preincubation are scattered around the same curve. This indicates that the rate of phosphate uptake is determined directly by the degree of acidification of the cells, rather than by the internal acid concentrations. The phosphate uptake rates found after incubation with caprylic acid appeared to be significantly smaller at given caproic acid distribution ratios than the uptake rates observed with the other fatty acids.

DISCUSSION

The extent of inhibition of anaerobic phosphate uptake observed after only 1 min preincubation with the fatty acid involved is much greater than the percentage inhibition found after 5 min of preincubation when considering a given internal fatty acid concentration. This is observed with all acids investigated except caprylic acid. Apparently, the cells are capable of protecting themselves against the inhibitory effect of the fatty acids. This was not observed earlier[3] when studying the effect of both butyric acid and formic acid upon phosphate uptake at 5 °C, which means that the recovery process has a high temperature coefficient. The recovery effect at 25 °C is accompanied by an increase in the caproate distribution coefficient, *i.e.* by an increase of the cell pH on which this distribution mainly depends, as shown in Fig. 1. Probably the recovery is due to a redistribution of ions *via* an enhancement of the cation proton pump induced by cell acidification. It has been shown by Lemmens in our laboratory that addition of butyric acid to the yeast cells leads to an increase in the rate of Rb^+ uptake, which lends support to this hypothesis.

A good correlation is found between the uptake rate of phosphate and the caproate distribution ratio. Therefore it is likely that inhibition of phosphate uptake by the fatty acids is due to a decrease in cell pH caused by these acids, rather than by a direct effect of the fatty acids upon some protein involved in the phosphate uptake process, a suggestion made by Samson *et al.*[1]. Our explanation is supported by the fact that the internal fatty acid concentrations needed for obtaining a certain degree of inhibition of phosphate uptake do not differ much for the varying fatty acids except for caprylic acid and caproic acid, whereas the affinity of fatty acids for proteins increases with increasing chain length[13]. The inhibition of several enzymic reactions by fatty acids is also much more effective with long-chain fatty acids than with short-chain fatty acids added in comparable concentrations[14,15]. The somewhat larger inhibition of phosphate uptake found with caprylic acid as compared with the other inhibitors, when considering a given decrease in caproate distribution ratio, may point to an additional specific effect of caprylic acid not directly related with the decrease in cell pH.

It was suggested by Borst-Pauwels and Jager[2] that phosphate is picked up by a specific acceptor at the outside of the cell membrane and that the phosphate–acceptor complex "reacts" with some compound Y, whereafter phosphate is accumulated into the cell As to the nature of Y, two possibilities were considered, namely, that Y is a high-energy compound formed anaerobically in the yeast or that Y is an unspecific anion carrier. For arguments against these two hypotheses, see refs 16 and 17, and ref 18, respectively. The results presented here point to a third possibility, namely, that Y is a factor closely related with the cell pH. Possibly there is some resemblance with the phosphate transport mechanism of animal mitochondria where phosphate can be exchanged for hydroxyl anions *via* an exchange diffusion system[19]

ACKNOWLEDGEMENT

We are grateful to the Gist- en Spiritusfabriek at Delft for providing us with the yeast.

REFERENCES

1 F E Samson, A M Katz and D L Harris, *Arch Biochem Biophys*, 54 (1955) 406
2 G W F H. Borst-Pauwels and S Jager, *Biochim Biophys Acta*, 172 (1969) 399
3 S Jager and G W F. H Borst-Pauwels, *Acta Bot Neerl*, 19 (1970) 147
4 A L Neal, J O Weinstock and J O Lampen, *J Bacteriol*, 90 (1965) 126
5 H Suomalainen and E Oura, *Exp Cell Res*, 9 (1955) 355
6 G W F H Borst-Pauwels, *FEBS Lett*, 1 (1968) 252
7 G W F H. Borst-Pauwels and J Dobbelmann, *Acta Bot Neerl*, 21 (1972) 38.
8 E Oura, H. Suomalainen and R Collander, *Physiol Plant*, 12 (1959) 534
9 W J Waddell and R G Bates, *Physiol Rev*, 49 (1969) 285
10 G W F H Borst-Pauwels, P L M. Huygen and J A Hoeberichts, in E Broda, A Locker, H Springer-Lederer, *Proc 1st Eur Biophys Congr*, Vol 3, 1971, p 225
11 A Kotyk, *Folia Microbiol*, 8 (1963) 27
12 J Goodman and A. Rothstein, *J Gen Physiol*, 40 (1957) 915
13 J D Teresi and J M Luck, *J Biol Chem*, 194 (1952) 823
14 S Dickstein, *Biochim Biophys Acta*, 36 (1959) 397
15 A P Nygaard, *J Biol. Chem*, 236 (1961) 2779
16 P L M Huygen, Thesis, Nijmegen, 1972
17 P L M Huygen and G W F H Borst-Pauwels, *Biochim Biophys Acta*, 283 (1972) 234
18 G W F H Borst-Pauwels and P L M Huygen, *Biochim Biophys Acta*, 288 (1972) 166
19 J B Chappell and A R Croft, in J M Tager, S Papa, E Quagliariello and E C Slater, *Regulation of Metabolic Processes in Mitochondria*, Elsevier Publ Co., Amsterdam, London, New York, 1966