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COMPARISON OF THE EFFECT OF ACIDIC INHIBITORS UPON ANAEROBIC PHOSPHATE UPTAKE AND DINITROPHENOL EXTRUSION BY METABOLIZING YEAST CELLS

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SUMMARY

The inhibition of anaerobic phosphate uptake by 2,4-dinitrophenol cannot be ascribed to competition of the two anions for the same anion carrier.

Pentachlorophenol, isooctyldinitrophenol and azide share the property with dinitrophenol in that they inhibit anaerobic phosphate uptake and dinitrophenolate efflux simultaneously.

The lower fatty acids, however, do not affect the dinitrophenolate efflux from yeast, when added in concentrations at which anaerobic phosphate uptake is inhibited.

INTRODUCTION

The distribution of 2,4-dinitrophenol between anaerobically metabolizing yeast cells and the medium is anomalous. The dinitrophenol, provided that it is added at low concentrations, is accumulated to a much lesser extent[1] than expected when only the undissociated acid penetrates the yeast cell[2,3]. This can be explained readily by assuming that the dinitrophenolate anion also passes the cell membrane. As a matter of fact, we have recently shown that nonmetabolizing yeast cells are slightly permeable to the anionic form of dinitrophenol[4]. The rate of anion efflux is increased considerably under metabolic conditions, and this efflux can be stopped by dinitrophenol itself in spite of the fact that the rate of glycolysis is decreased only slightly. Simultaneously with the inhibition of dinitrophenolate efflux, decreases in the rates of phosphate uptake occur[1]. This is observed both in the presence of dinitrophenol and when metabolism is stopped with iodoacetate.

It has been shown by us[5] that inhibition of anaerobic phosphate uptake by inhibitors of metabolism, fatty acids and 2,4-dinitrophenol can be attributed to the decrease of the concentration of an unknown compound Y which determined the rate of phsophate transfer from an acceptor–phosphate complex to the cell interior. The simultaneous inhibition of phosphate uptake and dinitrophenol efflux might indicate that not only the phosphate absorption rate but also the dinitrophenol efflux rate is determined by the unknown compound Y. One of the possibilities considered concerning the identity of compound Y was that it is a non-specific anion

carrier which operates only under metabolic conditions. Loading of the carrier by inhibitor anions which have been accumulated into the cell leads to a decrease in the effective carrier concentration and thus to an impairment of phosphate uptake. This view is in agreement with the finding that also phosphate efflux is inhibited by dinitrophenol[6]. In addition, the fact that the relative rate of dinitrophenolate efflux decreases on increasing the dinitrophenol concentration is readily explained by carrier saturation.

METHODS

The yeast *Saccharomyces cerevisiae*, strain Delft 2, was pre-aerated for 1 day at pH 4.5 in a 0.1 M sodium succinate buffer containing 10 mM KCl. The cells were then incubated anaerobically by bubbling nitrogen through a 1% (w/v) yeast suspension for 60 min at 25°C, either at pH 4.5 in the same buffer or at pH 6.8 in 0.1 M Tris–citrate buffer with 10 mM KCl, both in the presence of 3% glucose. The uptake of 10^{-6} M ^{14}C-labelled 2,4-dinitrophenol was studied as described before[1]. Inactive dinitrophenol or other inhibitors were added to the yeast together with the radioactive dinitrophenol. The yeast was centrifuged after 6 min, and 0.5 ml of the supernatant was assayed for radioactivity (A_{sup}) by means of liquid scintillation[1]. Also the radioactivity of 0.5 ml of the suspension (A_{sus}) was determined. The distribution ratio of radioactive dinitrophenol is given by

$$f = (A_{sus} - A_{sup})\, A_{sup}^{-1} \cdot V_e \cdot V_i^{-1}$$

where 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]. Azide, caprylate and dinitrophenol were added in buffer, whereas pentachlorophenol was added in methanol (final concentration, 5% methanol) and isooctyldinitrophenol was added as an alcoholic solution (final concentration, 5% ethanol). Concentrations of isooctyldinitrophenol and pentachlorophenol in the medium were determined in the supernatant obtained after 6 min incubation by measuring the absorbance with a spectrophotometer at 450 nm and 248 nm, respectively. The concentration of caprylate in the supernatant was determined in a parallel experiment carried out with [1-^{14}C]-caprylate. Initial rates of ^{32}P uptake were determined immediately after this 6-min period as described before[5] by adding KH_2PO_4, labelled with carrier-free ^{32}P-labelled phosphate to a final concentration of 0.1 mM, to the yeast suspension and filtrating 0.8-ml samples of the suspension at 10-s intervals during 1 min.

After preflushing the flasks with nitrogen, the rate of anaerobic glycolysis was determined by means of standard Warburg techniques, both in the presence and in the absence of appropriate amounts of inhibitor. Yeast cell pH values were determined as described in ref. 7.

RESULTS

Anaerobic phosphate uptake is inhibited by 50% by 0.022 mM dinitrophenol at pH 4.5 (ref. 5). This inhibition is not immediate but develops gradually in time and is maximal when the cellular dinitrophenol concentration does not increase any more[8]. This means that the low concentrations of dinitrophenol in the medium

do not affect anaerobic phosphate uptake but that the inhibition is due to dinitrophenolate anions accumulated into the cells. The concentration of cellular dinitrophenol at which anaerobic phosphate uptake is inhibited by 50 % is approximately 1 mM. The cell pH is about 6.80 under these conditions. We have now examined whether phosphate uptake is inhibited also when adding dinitrophenol at a pH equal to that of the cell water. If Y is a general anion carrier one would expect that this carrier can be loaded both by anions from the cell side and by anions from the medium side. Accumulation of dinitrophenol by the cells decreased dramatically on increasing the pH of the medium as shown before[4]. As a result, the probability that inhibition by accumulated anions into the cells will predominate over a possible inhibition by anions from the medium will be smaller than at a low medium pH. It appeared that concentrations of dinitrophenol up to 5 mM hardly affected the rate of anaerobic phosphate uptake at pH 6.8 (Table I).

TABLE I

THE EFFECT OF VARYING AMOUNTS OF DINITROPHENOL UPON THE KINETIC PARAMETERS OF PHOSPHATE UPTAKE AT pH 6.8

The numbers in parentheses represent the standard error of the mean. The values of the maximum rate (V) and the K_m were calculated by a digital computer using the Cleland program[9]. The yeast concentration was 1% (w/v).

Dinitrophenol (*mM*)	*V* ($mmoles \cdot min^{-1} \cdot kg^{-1}$)	K_m (μM)
0	3.92 (0.46)	27.9 (5.3)
3	3.49 (0.69)	21.7 (5.9)
4	3.57 (0.49)	20.7 (4.3)
5	3.82 (0.50)	24.0 (4.9)

We have examined whether other inhibitors of phosphate uptake which also decrease the concentration of compound Y affect the dinitrophenol efflux. It is seen in Fig. 1 that azide increased the distribution ratio of dinitrophenol (*i.e.* decreases the efflux) in just the same range of concentrations at which phosphate uptake is inhibited, whereas the rate of glycolysis is unaffected. Thus azide has an effect similar to that of dinitrophenol.

Similar results were obtained with pentachlorophenol. The concentration at which phosphate uptake was inhibited by 50 % by this uncoupler was 0.0011 mM. At this concentration the dinitrophenol distribution ratio was raised to 168. Fig. 2 shows that isooctyldinitrophenol also increased the accumulation ratio of dinitrophenol in the range of concentrations in which phosphate transport is inhibited, though initially to a somewhat lesser extent than observed with azide and pentachlorophenol at comparable rates of inhibition of phosphate uptake. An abrupt fall in the rate of glycolysis was seen at an inhibitor concentration of about 0.012 mM. The slope of the curve was then even slightly negative. This is due to the large increase in the isooctyldinitrophenol distribution ratio occurring in this range of concentrations, giving rise to a lower concentration of isooctyldinitrophenol in the medium at relatively high initial concentrations than at the lower ones. The distribu-

tion ratio of dinitrophenol increased, too. The accumulation of isooctyldinitrophenol, however, appeared to be much greater than that of dinitrophenol, indicating that the former compound is about 30 times more strongly adsorbed to yeast cell constituents than dinitrophenol[1].

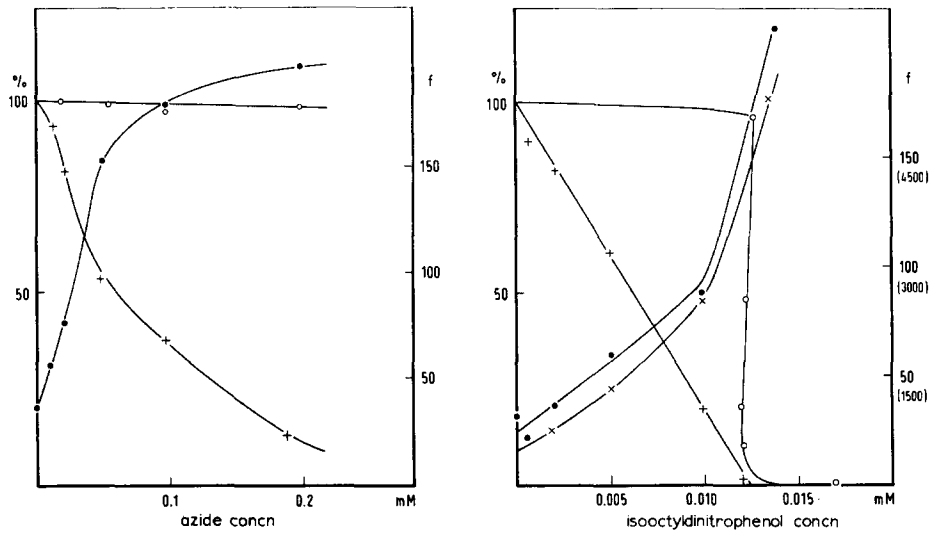

Fig. 1. Comparison of the effect of azide upon the distribution ratio of labelled dinitrophenol and upon the rates of anaerobic phosphate uptake and glycolysis. +, percentual rate of 10^{-4} M phosphate uptake at pH 4.5; ○, percentual rate of gycolysis; ●, distribution ratio of 10^{-6} M dinitrophenol. The yeast concentration was 1% (w/v). The concentrations of azide are initial concentrations. The rates of anaerobic phosphate uptake and of glycolysis are expressed in percents of the control (see scale on the left). The distribution ratio of dinitrophenol (f) is indicated on the right.

Fig. 2. Comparison of the effect of isooctyldinitrophenol upon the distribution ratios of dinitrophenol and of isooctyldinitrophenol with the percentual inhibition of anaerobic phosphate uptake and of glycolysis. ×, distribution ratio of isooctyldinitrophenol (values in parentheses). The concentration of isooctyldinitrophenol refers to the medium after the 6 min preincubation. The yeast concentration was 0.1% (w/v). See further legend to Fig. 1.

Caprylic acid did not affect the dinitrophenol accumulation ratio, as shown in Fig. 3, when added in the range of concentrations at which phosphate uptake is affected. A slight increase of the accumulation ratio was observed only at relatively high concentrations at which the rate of glycolysis was decreased, too. Similar results were obtained with butyric acid, caproic acid and acetic acid. We have examined whether a possible decrease in cell pH due to acidification of the cells[10] by the accumulated caprylic acid or to the increased fatty acid concentration in the cell counteracted the increase in accumulation of dinitrophenol. The fatty acid itself might compete with dinitrophenol for adsorption sites in the cell interior. Increasing amounts of inactive dinitrophenol were added together with 0.5 mM caprylic acid (initial concentration) to the yeast suspension, and the distribution ratio of radioactive dinitrophenol was determined. It is shown in Fig. 4 that the cells when loaded with caprylate extruded dinitrophenol, provided that the dinitrophenol concentration is sufficiently low.

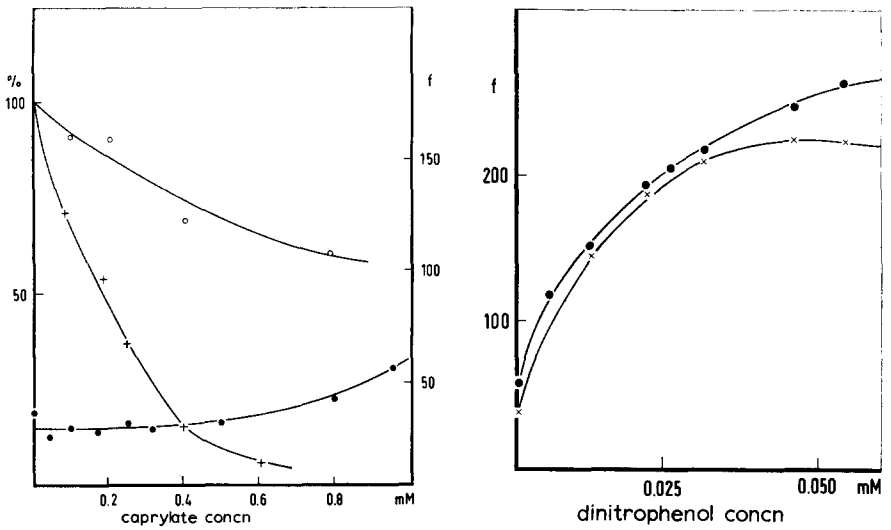

Fig. 3. Comparison of the effect of caprylate upon both dinitrophenol distribution ratio and the rates of anaerobic phosphate uptake and of glycolysis. The caprylate concentrations refer to the medium after the 6 min preincubation. The yeast concentration is 1% (w/v). See also legend to Fig. 1.

Fig. 4. Effect of dinitrophenol upon the dinitrophenol distribution ratio both in the presence and in the absence of 0.5 mM caprylate. ●, without caprylate; ×, with 0.5 mM caprylate (initial concentration). The dinitrophenol concentrations refer to the medium after the 6 min preincubation. The yeast concentration was 0.1% (w/v).

DISCUSSION

The fact that concentrations of dinitrophenol in the medium as high as 5 mM hardly affect the rate of anaerobic phosphate uptake at pH 6.8, a pH equal to that of the cell, shows that the affinity of the dinitrophenolate anion to a possible anion carrier for phosphate is much too small in order to account for the inhibition of phosphate uptake by dinitrophenol observed at pH 4.5. Under the latter conditions 50% inhibition of phosphate uptake is already found at a dinitrophenol concentration in the cell water amounting to only 1 mM. These results make it improbable that dinitrophenol interferes with the transport of phosphate *via* a decrease of the available sites of a hypothetical carrier. This does not rule out the possibility that the dinitrophenolate anion itself passes the cell membrane *via* a carrier system. It only shows that this carrier is not identical with Y, the unknown compound which determines the rate of anaerobic phosphate uptake[5]. In addition, inhibition of dinitrophenol efflux by dinitrophenol itself and other uncouplers or azide cannot be due to a decrease in Y. Fatty acids namely do not affect the efflux of dinitrophenol from the yeast cells under conditions when phosphate uptake is inhibited by these acids and compound Y is decreased simultaneously.

The inhibition of anaerobic phosphate uptake or dinitrophenol efflux by compounds like dinitrophenol and azide is not due to a decrease in the ATP level of the cells. The concentration of ATP does not decrease much in the presence of these compounds[11]. On the other hand, several energy-requiring processes like the synthesis

of RNA and proteins[11], the synthesis of polyphosphates[12] and the uptake of cations[12,13] are inhibited by dinitrophenol and azide in anaerobic yeast. In this connection it has been hypothesized that an unknown high-energy intermediate is formed in anaerobic yeast mediating the energy transduction from ATP to the processes involved[12]. Possibly this might also apply to the extrusion of dinitrophenolate from metabolizing yeast cells. However, it is obvious that if Y is the unknown high-energy intermediate the dinitrophenol extrusion cannot be mediated by this high-energy intermediate because the fatty acids which decrease the level of Y do not effect the dinitrophenol distribution between cells and medium.

For another possible explanation of the effect of the uncouplers and azide upon the dinitrophenolate fluxes across the yeast cell membrane, namely *via* a decrease of the membrane potential, we refer to refs 4 and 14.

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