BBA 45775

INHIBITION OF PHOSPHATE AND ARSENATE UPTAKE IN YEAST BY MONOIODOACETATE, FLUORIDE, 2,4-DINITROPHENOL AND ACETATE

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(Received November 19th, 1968)

SUMMARY

1. Arsenate and phosphate uptake by the yeast *Saccharomyces cerevisiae* Delft II is inhibited completely by 3 mM monoiodoacetate, 20 mM fluoride, 0.1 mM 2,4-dinitrophenol and 60 mM acetate under anaerobic conditions at pH 4.5.

2. Monoiodoacetate, 2,4-dinitrophenol and acetate decrease not only the maximum rate of uptake but also the K_m for this process.

3. The percent decrease in the rates of glycolysis and phosphate uptake caused by monoiodoacetate is about the same. The three other inhibitors inhibit glycolysis only at concentrations much higher than those which decrease phosphate uptake.

4. The cellular ATP concentration is strongly decreased by 3 mM monoiodoacetate and 20 mM fluoride (90%), but much less by 0.1 mM 2,4-dinitrophenol (60%) and by 60 mM acetate (25%).

5. Possible ways in which the inhibitors may affect P_i and As_i uptake are discussed.

INTRODUCTION

Phosphate uptake by yeast depends upon metabolism as shown by HEVESY, LINDERSTRØM-LANG AND NIELSEN[1] and by MULLINS[2] among others. The dependence of the rate of phosphate uptake upon the concentration can be described by a Langmuir isotherm. In the context of ion transport this suggests that we are dealing with a carrier- or enzyme-mediated mechanism. This idea is supported by the finding that yeast cells are generally impermeable to phosphate as found by HEVESY, LINDERSTRØM-LANG AND NIELSEN[1], by SWENSON[3], and by SCHÖNHERR AND BORST-PAUWELS[4]. Two uptake mechanisms can be operative: one with a low affinity for phosphate, having a K_m of about $5 \cdot 10^{-4}$ M (GOODMAN AND ROTHSTEIN)[5], and one with a K_m of 10^{-5} M (BORST-PAUWELS)[6]. LEGGETT[7] showed that both mechanisms can occur simultaneously in yeast as was also found in plant roots by HAGEN AND HOPKINS[8]. Blocking of phosphate uptake in yeast by 2,4-dinitrophenol under aerobic conditions was found by HOTCHKISS[9] and also by NICKERSON AND MULLINS[10]. LEGGETT also observed that monoiodoacetate and 2,4-dinitrophenol under anaerobic conditions inhibit phosphate

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uptake. The inhibition by monoiodoacetate is of the non-competitive type. HAGEN AND HOPKINS showed that dinitrophenol is a competitive inhibitor of phosphate uptake in plant roots. We shall present evidence of an entirely different type of inhibition of phosphate uptake in yeast and discuss its possible implications for the interpretation of the uptake mechanism. In addition we studied the effects of the inhibitors upon the rate of glycolysis and the cellular ATP concentration in order to obtain information concerning the relation between phosphate uptake and energy metabolism. The effects of the inhibitors upon the uptake of arsenate are also examined because arsenate is accumulated by the same mechanism (ROTHSTEIN)[11], but is not incorporated into organic compounds or polyanions (BORST-PAUWELS *et al.*)[12]. Therefore a comparison of effects of inhibitors upon phosphate uptake and arsenate uptake may answer the question whether the inhibitors affect membrane transfer of phosphate or the incorporation of phosphate into other compounds after passage through the membrane.

METHODS

The yeast *Saccharomyces cerevisiae* Delft II is preaerated for at least 1 day in a 0.1 M sodium succinate buffer of pH 4.5 provided with 9.4 mM KCl and 39 mM ammonium sulphate in order to exhaust the internal substrates. Studies on the effects of inhibitors upon the rate of P_i or As_i uptake are carried out according to the following scheme: 1.1 % (w/v) yeast is preincubated for 1 h in the presence of 30 g/l glucose under anaerobic conditions. This procedure increases the rate of P uptake in the pH 4.5 buffer greatly. To 1 vol. of this yeast suspension 0.1 vol. of succinate buffer (pH 4.5), with or without inhibitors, is added. After 6 min (for monoiodoacetate also 12 min) 9.9 vol. of the same buffer are added containing [^{32}P]phosphate or [^{74}As]-arsenate (from 1 to 20 μM and in a few cases 100 μM final concentration) and the appropriate inhibitor in the same concentrations as previously present.

Tracer incorporation is measured by removing a 0.8-ml sample every 6 sec during 1 min. These samples are filtered by water suction over Schleicher and Schüll No. 602 h paper in a Hirsch funnel, then rapidly washed twice with 2 ml of water of 0° and once with 3 ml of acetone. The radioactivity of the yeast on the filter paper is determined by an end-window Geiger–Müller tube. The slope of the uptake curve at zero time is taken as a measure for the initial rate of uptake. The inhibitor 2,4-dinitrophenol rapidly enters the cell. Its concentration in the medium after 6 min preincubation was greatly reduced. Hence the dinitrophenol added to the tracer medium was adjusted to this final concentration. Concentrations of 2,4-dinitrophenol in the supernatant are determined by measuring the absorbance at 360 mμ with a Zeiss spectrophotometer. The amount of dinitrophenol absorbed by the cells is calculated from the decrease in absorbance.

For the determination of the ATP concentration 2 vol. of 1 % yeast suspension are added to 1 vol. of 15 % $HClO_4$. The mixture is kept at 40° for 15 min (ESTABROOK AND MAITRA)[13], and the extract is subjected to a fluorimetric determination of the reduction of $NADP^+$ to NADPH in the presence of glucose, hexokinase, glucose-6-phosphate dehydrogenase and Mg^{2+} at pH 7.5 (GREENGARD)[14]. Appropriate corrections are made for glucose 6-phosphate initially present. Dinitrophenol, if present, is removed from the perchloric acid extract by repeated ether extraction, since its

yellow colour interferes with the fluorimetric NADPH determination. Fluoride, monoiodoacetate and acetate did not affect the ATP determinations in the concentrations used. The rate of anaerobic glycolysis expressed in mmoles CO_2 per min, and kg dry wt. of yeast is determined by the usual manometric techniques.

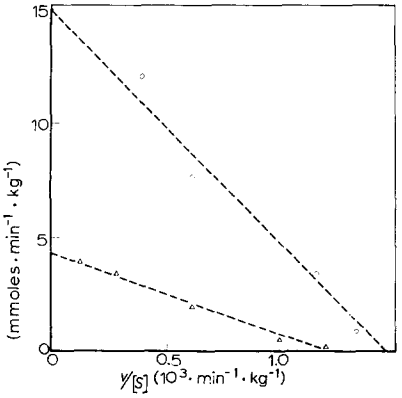
RESULTS

Michaelis-Menten constants and maximum rates of uptake of phosphate or arsenate were determined from plots of the initial rate of uptake (v) and the quotient of the initial rate and the concentration of phosphate or arsenate ($v/[S]$) (DIXON AND WEBB)[15]. According to equation (1) the slope of the straight line found will be equal to $-K_m$, and the intercept with the Y-axis is equal to V:

$$v = V - K_m \frac{v}{[S]} \tag{1}$$

The plot of v versus $v/[S]$ always yielded straight lines (Fig. 1). Therefore, we are dealing with phosphate uptake *via* one transport mechanism only, which has a K_m of about 10^{-5} M. The values of V, K_m, ATP concentration and glycolytic rate (v_g) in the absence of inhibitor are as follows: The mean values (12 determinations) for the uptake of P_i were V, 15.6 ± 1.0 (standard error) mmoles·min^{-1}·kg^{-1} (dry wt.); K_m, 13.7 ± 0.9 μM, and for As_i (4 determinations): V, 13.3 ± 1.2 mmoles·min^{-1}·kg^{-1}; K_m, 15.5 ± 2.3 μM. The concentration of ATP was 3.75 ± 0.25 mmoles·kg^{-1} (4 determinations). The rate of glycolysis was 180 ± 9 mmoles CO_2·min^{-1}·kg^{-1} (8 determinations).

Fig. 1 shows the effect of dinitrophenol upon the concentration dependence of phosphate uptake. It is seen that both the slope of the straight line and the intercept with the Y axis are decreased after addition of the inhibitor. This means that both K_m and V decrease. The effects of dinitrophenol upon V, K_m, v_g and C_{ATP}, expressed in percent of the control, are compared in Fig. 2. The rate of glycolysis is only slightly



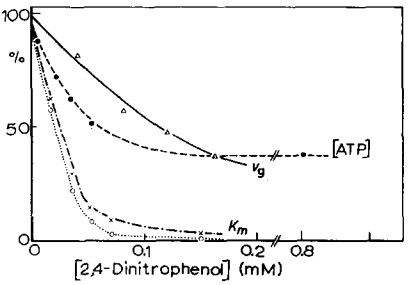


Fig. 1. Plot of the initial rate of phosphate uptake (v) against the quotient of this rate and the phosphate concentration ($v/[S]$). The effect of 6 min preincubation with 0.03 mM 2,4-dinitrophenol (△). ○, control.

Fig. 2. Effects of 2,4-dinitrophenol upon the kinetic data for P_i uptake expressed in percent of control values. Final dinitrophenol concentrations observed in the supernatant of the yeast are plotted. v_g is the rate of anaerobic CO_2 production.

diminished under conditions where phosphate uptake is almost completely inhibited. The relative decrease in ATP, though larger than that of v_g, is also smaller than the relative decrease in V.

Table I shows the effect of monoiodoacetate upon V, K_m, glycolytic rate (v_g) and cellular ATP concentration. It is seen that the relative decreases in V and v_g are about the same. The percent decreases in C_{ATP} and K_m are only slightly smaller. In contrast to the findings of LEGGETT[7] we observed a decrease in K_m, too. This difference may be due to the fact that we are dealing with initial rates of uptake, whereas LEGGETT determined average rates of absorption over several minutes. The effects of monoiodoacetate appeared to be much more pronounced after 12 min than after 6 min preincubation.

Fluoride appeared to inhibit phosphate uptake much more than glycolysis. While K_m decreased by only 30% upon increasing fluoride concentration, V decreased to almost zero, paralleling the decrease in cellular concentration of ATP (Fig. 3).

Acetate can also inhibit anaerobic phosphate uptake in yeast, as previously shown by SAMSON, KATZ AND HARRIS[16]. Anaerobic CO_2 production, which is probably due mainly to glycolysis, though a small part may also be caused by anaerobic oxidation of acetate[17], is much less affected than P_i uptake in this case (Fig. 4).

In our experiments K_m was also found to decrease. The concentration of ATP did not decrease much, even at concentrations of acetate at which glycolysis was largely blocked.

The corresponding values of K_m and V from all experiments are plotted in Fig. 5. A linear relationship is found for the control experiments and all inhibitors,

TABLE I

EFFECTS OF MONOIODOACETATE UPON KINETIC PARAMETERS OF PHOSPHATE UPTAKE IN PERCENT OF CONTROL VALUES

	6-min preincubation				*12-min preincubation*			
	V	K_m	C_{ATP}	v_g	V	K_m	C_{ATP}	v_g
1 mM monoiodoacetate	74	80	77	77	24	38	34	24
3 mM monoiodoacetate	19	33	32	15	7	17	16	8

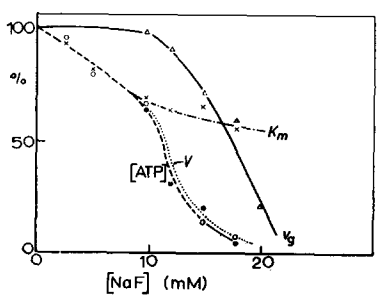


Fig. 3. The effect of fluoride upon the kinetic data for P_i uptake expressed in percent of control values. Initial inhibitor concentrations are plotted.

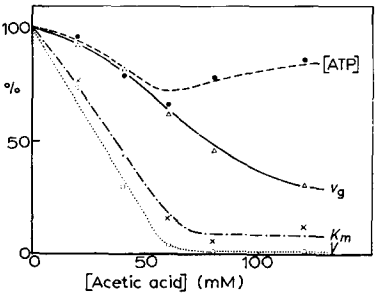


Fig. 4. The effect of acetate upon the kinetic data for P_i uptake expressed in percent of the control. Initial inhibitor concentrations are plotted.

except fluoride, with an intercept with the Y-axis of about 10^{-6} M. The deviating behaviour of fluoride is apparent from this figure.

Fig. 6 shows the relation between the V values found in the different experiments, and the corresponding ATP concentrations. No single relation exists between V and C_{ATP}. Monoiodoacetate and NaF give rise to an almost proportional decrease in V. Acetate and dinitrophenol, however, cause a much larger decrease in V than in C_{ATP}.

A plot of V against the rate of anaerobic CO_2 production which presumably does not differ much from the rate of glycolysis (v_g) is shown in Fig. 7. An almost linear relationship between these quantities is found for the experiments with monoiodoacetate as inhibitor. Fluoride, acetate and dinitrophenol, however, do not exhibit

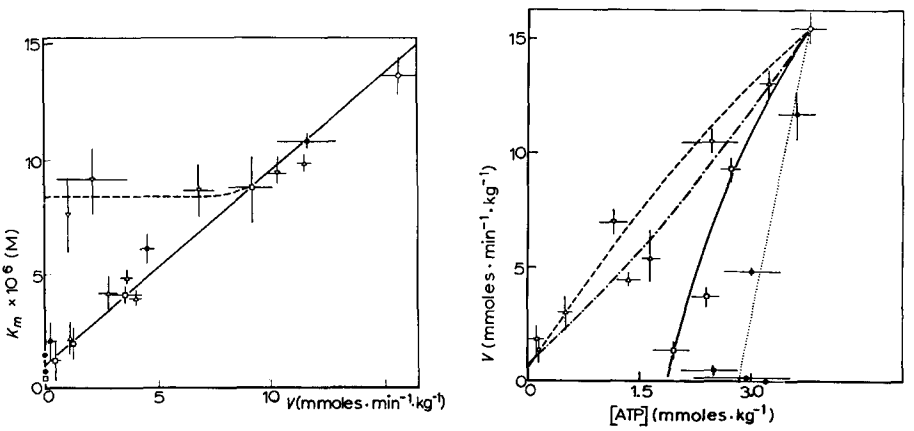


Fig. 5. Plot of K_m for phosphate uptake against the corresponding values of V observed in experiments with monoiodoacetate, fluoride, acetate and dinitrophenol. ◇, no inhibitor; ●, acetic acid; □, 2,4-dinitrophenol; △, monoiodoacetate; ▽, NaF.

Fig. 6. Plot of V for phosphate uptake against intracellular ATP concentration found in experiments with monoiodoacetate, fluoride, acetate, and 2,4-dinitrophenol. For symbols see Fig. 5.

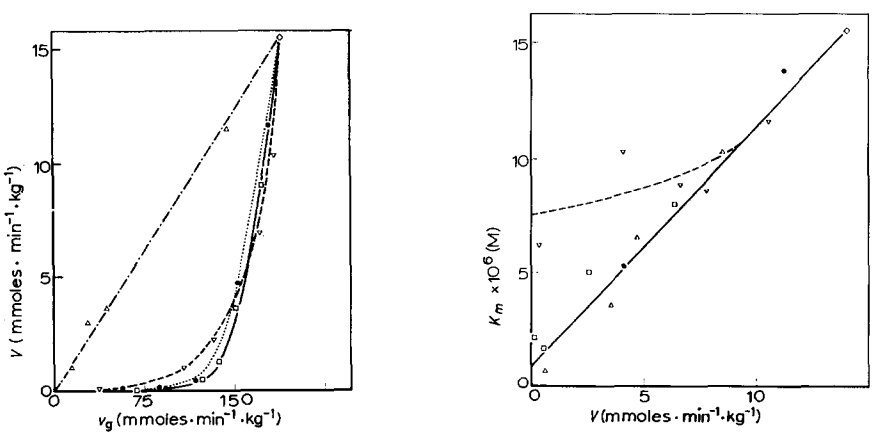


Fig. 7. Plot of V for phosphate uptake against rate of anaerobic CO_2 production (v_g) for experiments with monoiodoacetate, fluoride, acetate and dinitrophenol. For symbols see Fig. 5.

Fig. 8. Plot of K_m for arsenate uptake against corresponding V values from experiments with monoiodoacetate, fluoride, acetate and dinitrophenol. For symbols see Fig. 5.

such a relationship: there is only a slight decrease in v_g, while V decreases to 20% of the control value.

The inhibitors affected phosphate uptake in about the same way when a 0.1 M citrate buffer was used instead of the 0.1 M succinate buffer. This is important in view of the observation of IDZIAK[15] that yeast is not quite impermeable to succinate, whereas citrate does not enter the cell at all.

The results of the experiments with arsenate were quite similar to those with phosphate. There are no significant differences in K_m or V for phosphate and arsenate uptake. The inhibitors lowered V for As uptake to about the same extent as for P_i uptake. Fluoride decreased K_m only to about $8 \cdot 10^{-6}$ M, whereas the other inhibitors gave rise to a much larger decrease in K_m. Fig. 8 shows the relation between K_m and V for arsenate uptake.

We have also investigated whether changes in the time of preincubation affected the Michaelis-Menten constant of P_i uptake. An increase in the time of preincubation with substrate leads to an increase in the maximum rate of uptake (LEGGETT[7], BORST-PAUWELS[19]). Upon increasing the preincubation time from 66 min to 180 min in the presence of 3% glucose under anaerobic conditions, V was raised from 15.6 mmoles$\cdot$min$^{-1}\cdot$kg^{-1} (dry wt.) to 42 mmoles$\cdot$min$^{-1}\cdot$kg^{-1}, whereas K_m did not change at all. Therefore changes in V are not necessarily accompanied by variations in K_m.

DISCUSSION

The qualitative and quantitative similarity between the results obtained with phosphate and arsenate seems to exclude the possibility that the effects observed are due to an interference of the inhibitors with the metabolism of the accumulated phosphate. Therefore we are dealing with effects of the inhibitors on the mechanism of phosphate or arsenate transport across the cell membrane. In view of the similarity in arsenate or phosphate uptake, we shall only discuss the mechanism of phosphate uptake here. The conclusions, however, should also be valid for arsenate uptake. The observed inhibition of phosphate uptake by the metabolic inhibitor monoiodoacetate is in accordance with the general view that phosphate absorption is dependent upon metabolism. A dependence of phosphate absorption upon glycolysis has been observed by GOODMAN AND ROTHSTEIN[5]. Decrease of the free concentration of Mg^{2+} in the yeast cell leads to a parallel decrease in glycolysis and phosphate uptake (BORST-PAUWELS)[20]. Contrary to the effects of monoiodoacetate, fluoride appeared to be capable of inhibiting phosphate uptake to a large extent without greatly affecting glycolysis. Only at concentrations above 10 mM glycolysis is inhibited as well. This shows that the coupling between metabolism and phosphate uptake is not tight. The possibility that ATP supplies the energy necessary for P_i accumulation is supported by the observation that monoiodoacetate and fluoride decrease the content of ATP in the cell to about the same extent as the maximum rate of phosphate uptake.

Acetate and dinitrophenol, however, are able to block the phosphate uptake almost completely without much decreasing the ATP concentration. This is especially apparent in the case of acetate. It is not very probable that the action of acetate on P_i uptake is due to a decrease in intracellular pH. Caprylic acid gives almost the same results as observed with acetic acid, though at a hundred times lower concentration (JAGER)[21].

The existence of an unknown high-energy compound related to anaerobic processes in yeast was considered by RIEMERSMA[22] in a study of the effect of dinitrophenol and azide on proton secretion and by JARETT AND HENDLER[23] studying the interaction of the same inhibitors on protein and RNA synthesis. The question of whether the effects of acetate and dinitrophenol on P_i uptake under anaerobic conditions are also due to the decrease of the concentration of such a hypothetical high-energy intermediate is the object for further research.

Generally the assumption is made that ions are taken up in plant cells by means of carriers, which are transformed at the inner side of the cell membrane into compounds having a low affinity for the ion involved. The rate of uptake can still be described by enzyme kinetics provided that the concentration of the carrier is kept constant by a regenerative process (EPSTEIN AND HAGEN)[24]. The similarity of the type of inhibition of phosphate uptake observed with the different inhibitors except fluoride may be an indication that the same step of the phosphate transport process is affected by these inhibitors. A linear relationship between K_m and V as observed by us (Fig. 5) is a common phenomenon encountered in enzymology. It may indicate that the rate of breakdown of the enzyme-substrate complex to free enzyme and product depends upon the concentration of an additional compound ("Y") which reacts with this complex. Decreases in the concentration of this compound will lead to decreases in both V and K_m provided that the rate of breakdown is not very small relative to the velocity of the establishment of the equilibrium of substrate and enzyme[25]. The extrapolated value of K_m for $V = 0$ is equal to the dissociation constant of the enzyme–substrate complex. Apparently this value is about 10^{-6} M in our case. We can only speculate about the nature of Y. Possibly it is a high-energy compound like ATP or some unknown high-energy intermediate, and its hydrolysis provides the decrease in free energy necessary for the accumulation of orthophosphate. When Y is ATP then one has to assume that acetate and partly also dinitrophenol prevent the energy transduction from ATP to the carrier system. In connection with discussions by VAN DAM AND SLATER[26] concerning the existence and role of a nonspecific anion carrier in mitochondria which is able to transfer not only carboxylic acids but also dinitrophenol and possibly also orthophosphate, we have considered whether the results observed by us can also be explained by a kind of competition of the inhibitors used and phosphate for the same carrier. This should be possible when Y represents the carrier and when orthophosphate is at first bound by a specific permease which transfers orthophosphate to that carrier.

Apparently fluoride decreases K_m to a certain extent only. This may indicate that the concentration of Y is not decreased as much by fluoride as by the other inhibitors. Another possibility is that fluoride works *via* an all or none type of inhibition. Then there would be a critical concentration of about 10 mM below which only a slight inhibition occurs, due to a decrease in the concentration of Y, and above which the inhibition is nearly complete. If the critical concentration differs somewhat from cell to cell, then the K_m observed would be mainly determined by the uptake of phosphate by uninhibited cells. The maximum rate of uptake and also the mean concentration, however, will depend equally upon the corresponding values in all cells. The remarkably sharp break occurring at about 10 mM in the curves for V, v_g and C_{ATP} supports this view.

The way in which acetate and partly also dinitrophenol inhibit the hydrolysis

of cell ATP is unknown to us. High concentrations of dinitrophenol can inhibit animal mitochondrial ATPases[27]. We are now investigating whether the yeast cell ATPases are also inhibited by dinitrophenol and possibly also by acetate.

The time of preincubation does not seem to affect the concentration of Y, because only V increases and not K_m. This would mean that the number of sites binding phosphate initially increases during the pretreatment with glucose.

ACKNOWLEDGEMENTS

The authors are much indebted to Prof. Dr. S. L. BONTING and his staff of the Laboratory of Biochemistry at Nijmegen and to Prof. Dr. CH. M. A. KUYPER of this laboratory for their critical remarks. The expert technical assistance of Mr. J. HENRICKS was greatly appreciated. The yeast was kindly provided by the "Gist- en Spiritusfabriek" at Delft.

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