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THE INTERACTION OF 2,4-DINITROPHENOL WITH ANAEROBIC Rb^+ TRANSPORT ACROSS THE YEAST CELL MEMBRANE

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SUMMARY

1. The Rb^+ uptake mechanism of yeast is activated by small amounts of Rb^+ or K^+, giving rise to a deviation from normal Michaelis–Menten kinetics at concentrations below 0.6 mM Rb^+.

2. The affinity of Rb^+ and K^+ for the activation sites appeared to be much higher than for the substrate sites.

3. 2,4-Dinitrophenol inhibits the Rb^+ uptake by lowering the affinity for both the activation sites and the substrate sites.

4. 2,4-Dinitrophenol causes a sudden increase in the Rb^+ permeability of the cells. This increase is only transient except at relatively high dinitrophenol concentrations.

INTRODUCTION

It has been shown by RIEMERSMA[1] that 2,4-dinitrophenol inhibits cation transport by yeast. The type of inhibition, however, was not examined. Recently HAROLD AND BAARDA[2] reported that Rb^+ uptake by *Streptococcus faecalis* is inhibited competitively by uncouplers of oxidative phosphorylation, and that phosphate uptake is inhibited *via* a non-competitive type of inhibition. In their experiments cation uptake was decreased more at lower concentrations and phosphate uptake more at higher concentrations. We also found that anaerobic phosphate uptake by yeast is inhibited by dinitrophenol more effectively at higher phosphate concentrations than at lower ones[3]. This made us consider whether the inhibition of Rb^+ uptake by yeast cells is also of the competitive type.

In addition, the effect of dinitrophenol upon cell permeability for Rb^+ was studied. According to RIEMERSMA[4] dinitrophenol increases the permeability of resting cells to K^+. When this is also true for metabolizing cells, then impairment of active cation transport might be compensated partly by an increased inward diffusion into the cells, thus complicating the evaluation of the type of inhibition.

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MATERIALS AND METHODS

The yeast *Saccharomyces cerevisiae* Delft II is pre-aerated for 1 day in distilled water, then centrifuged and re-suspended in 45 mM Tris–succinate buffer at pH 4.5 and 25° in the presence of 3 % glucose. N_2 is bubbled through the suspension. The yeast concentration is 2.2 % (w/v). After 1 h the suspension is diluted to 2.1 % by means of buffer with or without inhibitor. Radioactive Rb^+ ($^{86}RbCl$ from Amersham, Great Britain), diluted with appropriate amounts of inactive RbCl, is added 6 min later to the suspension at a final concentration of 0.02 μC/ml. The concentration of the suspension is then 2 % (w/v). The Rb^+ uptake in the cells is measured by removing a 0.8-ml sample at appropriate times. These samples are filtered by water suction over Schleicher and Schüll No. 602h 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 means of an end-window Geiger–Müller counter.

Pre-loading of the cells with radioactive Rb^+ or Na^+ ($^{22}NaCl$ from Philips Duphar, The Netherlands) is carried out by incubating 4 % yeast (w/v) in the presence of 3 % glucose in 45 mM Tris–succinate buffer with either carrier-free $^{86}RbCl$ or $^{22}NaCl$ added to a final concentration of 0.2 μC/ml. Efflux of the isotopes from the cells into the medium is determined after adding varying amounts of 2,4-dinitrophenol to the yeast and also KCl and NaCl to a final concentration of 25 mM and 50 mM, respectively. The yeast is diluted to 3.6 % (w/v). The salts are added in order to prevent re-entry of the released isotopes. 0.8-ml samples are taken from supernatants obtained at appropriate times by centrifuging the suspension. The samples are dried and the radioactivity is determined by means of a Geiger–Müller counter.

The rate of anaerobic glycolysis is determined by means of common manometric methods. The manometers are pre-flushed with nitrogen.

Concentrations of K^+ in the medium are determined by means of flame photometry. The concentration of dinitrophenol in the medium is determined by measuring the absorbance at 360 nm.

RESULTS

The kinetics of Rb^+ uptake by yeast and the effect of 2,4-dinitrophenol upon this process are shown in Fig. 1 for both a relatively low and a relatively high Rb^+ concentration. The uptake curve observed at 8 mM Rb^+ appeared to deviate from a straight line. Initial uptake rates were obtained from the slope of the curve at time zero. Apparently the inhibition of Rb^+ uptake by dinitrophenol observed at 0.5 mM Rb^+ is overcome for the greater part by increasing the Rb^+ concentration to 8 mM. The concentration dependence of Rb^+ uptake by yeast can normally be described by Michaelis–Menten kinetics[5,6]. Then a plot of the initial rate of uptake (v) against the quotient of this rate and the cation concentration ($v/[S]$) will give rise to a straight line according to Eqn. 1 (ref. 7).

$$v = v_{max} - K_m(v/[S]) \quad (1)$$

It is seen from Fig. 2 that deviations from linearity occur especially at lower Rb^+ concentrations. The uptake rate is smaller than expected according to Eqn. 1. As a

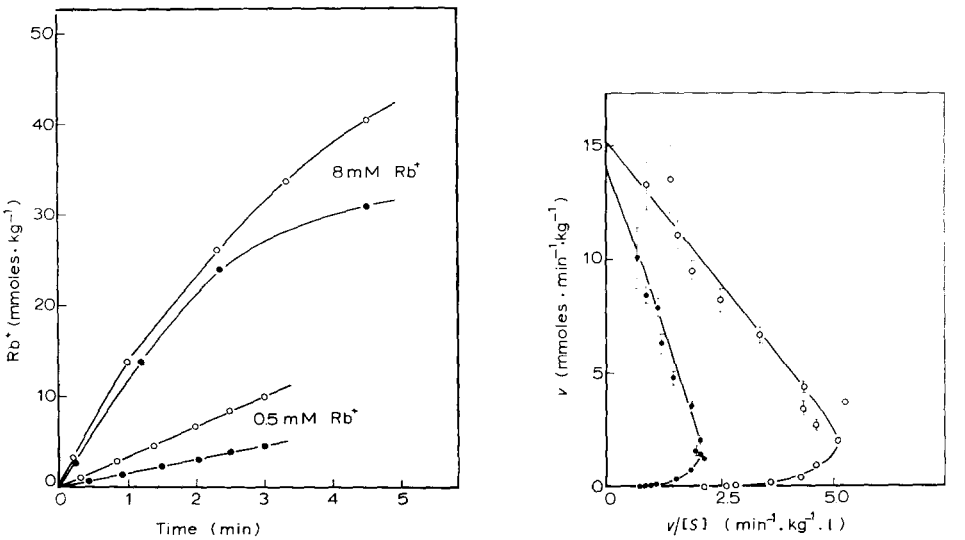

Fig. 1. The time-course of Rb^+ uptake at both 0.5 mM Rb^+ and 8 mM Rb^+ and the effect of 0.06 mM dinitrophenol upon this uptake. ○, without dinitrophenol; ●, with dinitrophenol.

Fig. 2. Plot of the rate of uptake (v) *versus* the quotient of the rate of uptake and the concentration ($v/[S]$). v is related to the dry weight of the yeast. Same symbols as in Fig. 1. The standard errors of v are indicated in the figures.

TABLE I

COMPARISON OF THE EFFECT OF LOW CONCENTRATIONS OF K^+ AND OF Rb^+ UPON THE UPTAKE RATE OF CARRIER FREE ^{86}Rb

Concentration of added cation (mM)	*^{86}Rb uptake rate (% of control)*			
	No dinitrophenol		*0.06 mM dinitrophenol*	
	+ K^+	*+ Rb^+*	*+ K^+*	*+ Rb^+*
No addition	100	100	100	100
0.015	144	141	105	114
0.052	149	166	135	133
0.100	185	183	144	141

matter of fact a sigmoid relation is found between the uptake rate and the Rb^+ concentration which points to an activation of the Rb^+ transport mechanism by Rb^+ itself. Table I shows that K^+ is almost as effective as Rb^+ in activating the Rb^+ uptake mechanism. Competition between labelled Rb^+ and inactive Rb^+ or K^+ for the substrate sites is still very small within the concentration range applied (see refs. 5 and 6). The Rb^+ uptake can be described by a mechanism analogous to that observed in enzymic processes when the activating ion combines with the enzyme independently of the substrate[7], see Eqn. 2. The concentration of the activating ion ($[a]$) should then be replaced by the sum of the concentrations of Rb^+ ($[S]$) and K^+ ($[S']$). Some K^+ is always present in the medium because of the establishment of an equilibrium between cells and medium[8].

$$v = \frac{v_{max}[S]}{K_m + [S]} \cdot \frac{[a]}{K_a + [a]} = \frac{v_{max}[S]}{K_m + [S]} \cdot \frac{[S] + [S']}{K_a + [S] + [S']} \quad (2)$$

K_a is the activation constant.

Eqn. 2 can be transformed into Eqn. 3 which is more suitable for the evaluation of the kinetic constants.

$$\frac{v}{(v_{max} - v)([S] + [S'])} = \frac{1}{K_m + K_a} \cdot \frac{K_m K_a + K_m[S']}{K_m + K_a} \cdot \frac{v}{(v_{max} - v)([S] + [S'])[S]} \quad (3)$$

The values of v_{max} are obtained from Fig. 2. According to Eqn. 1 which holds approximately at higher Rb^+ concentrations v_{max} is equal to the intercept with the ordinate. The values of $[S']$ are given in Table II. Plots according to Eqn. 3 are made in Fig. 3. The points lie approximately on a straight line showing that the Rb^+ uptake can be described very well by the activation model. Values of K_m and K_a (see Table II)

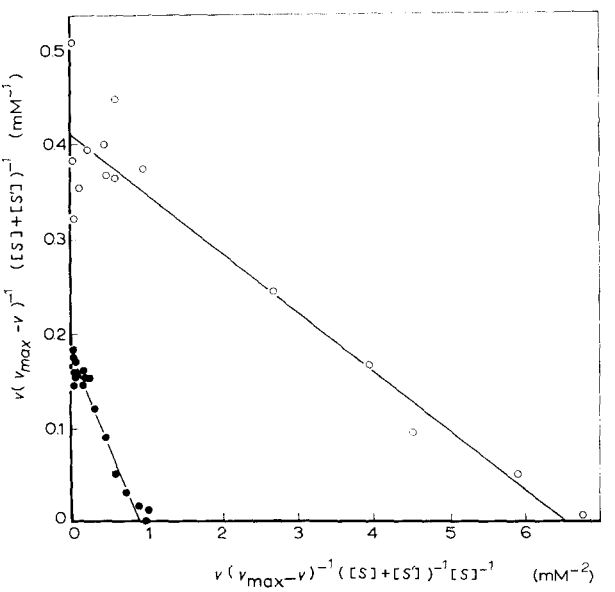

Fig. 3. Plot of the data of Fig. 2 according to Eqn. 3. The symbols are the same as those used in Fig. 1.

TABLE II

KINETIC DATA FOR Rb^+ UPTAKE AND THE EFFECT OF 2,4-DINITROPHENOL ON THIS UPTAKE

The activation constant K_a is defined by Eqn. 2 and refers to the sum of added Rb^+ (S) and already present K^+ (S'). The maximum rate of uptake v_{max} refers to the dry weight of the yeast. The concentration of dinitrophenol in the medium decreased to about 0.03 mM after the 6 min preincubation period due to its accumulation by the cells[9].

Addition	v_{max} (*mmoles* · $min^{-1} \cdot kg^{-1}$)	K_m (*mM*)	K_a (*mM*)	$[S']$ (*mM*)
Control	15.0	2.43	0.045	0.020
0.06 mM dinitrophenol	14.0	5.46	0.161	0.050

are calculated from the values of the slope of the straight line and the values of the intercept with the ordinate by solving the equations of the slope and the intercept.

We have examined the effect of increasing the Rb^+ concentration on the rate of anaerobic glycolysis in order to see whether the activation of cation uptake might be due to a stimulation of the rate of glycolysis[10]. It appears from Fig. 4 that the rate

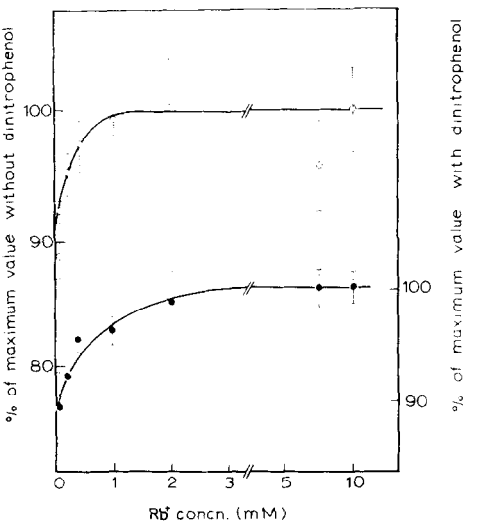

Fig. 4. Concentration dependence of the rate of glycolysis expressed as a percentage of the value found at full stimulation and the effect of 0.06 mM dinitrophenol upon it. Same symbols as in Fig. 1.

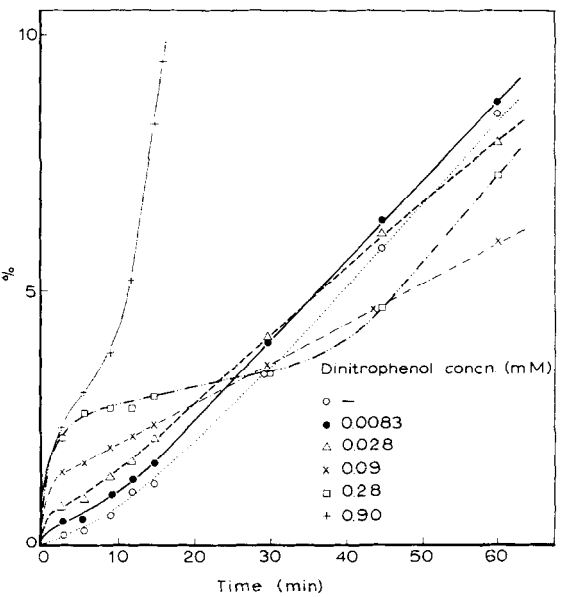

Fig. 5. Outflow of Rb^+ from cells pre-loaded during 1 h with carrier-free $^{86}Rb^+$, and the effect of varying amounts of dinitrophenol upon this release. The outflow is expressed as a percentage of the initially present radioactivity in the cells.

of glycolysis in the absence of Rb^+ already amounts to 90 % of the maximal value. 2,4-Dinitrophenol inhibits glycolysis by 12 % only. The activation of glycolysis by RbCl is counteracted by dinitrophenol just as is found with the activation of cation uptake. The amount of Rb^+ necessary for increasing glycolysis from 90 to 95 % of the maximal value is 0.20 mM without and 0.45 mM with 2,4-dinitrophenol present.

The effect of 2,4-dinitrophenol upon the Rb^+ permeability of metabolizing cells is examined by determining the Rb^+ efflux from pre-loaded cells (see Fig. 5). Dinitrophenol stimulates the Rb^+ outflow only transiently, and the subsequent release is even slower than found in the absence of dinitrophenol except at 0.9 mM dinitrophenol. At 0.28 mM dinitrophenol a sudden increase in the rate of outflow is observed after 40 min incubation with the inhibitor.

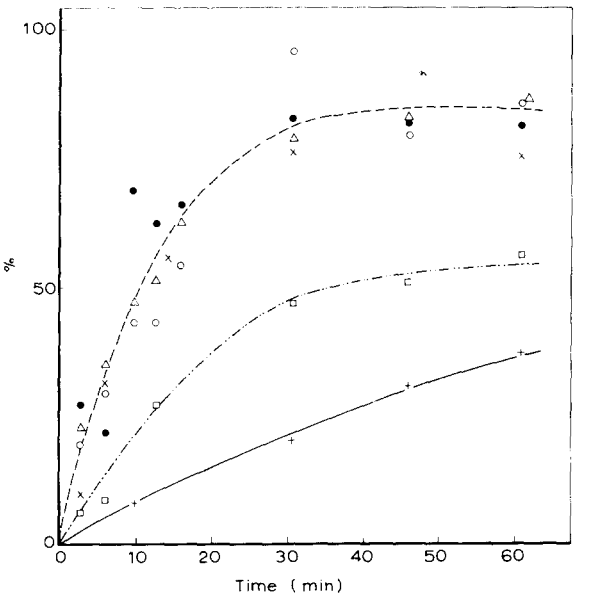

Fig. 6. Outflow of Na^+ from cells pre-loaded with carrier-free $^{22}Na^+$, and the effect of dinitrophenol upon this release. See also subscript to Fig. 6. The meaning of the symbols is the same as those of Fig. 5.

The effect of dinitrophenol on Na^+ release is shown in Fig. 6. The efflux rate of labelled Na^+ from pre-loaded cells expressed in percents of the amount initially present in the yeast released per min is about 100 times larger than the Rb^+ efflux rate being 7.5 %·min^{-1} and 0.08 %·min^{-1}, respectively. A transient increase of the outflow rate is not observed with Na^+ on adding dinitrophenol. The efflux is decreased significantly by 2,4-dinitrophenol concentrations higher than 0.1 mM. No increase of the efflux rate was observed after 40 min incubation with 0.28 mM dinitrophenol as was found for Rb^+ release. Nor was there an immediate increase found with 0.9 mM dinitrophenol.

It might be questioned whether the effects of dinitrophenol described in this article are due only to its acidic nature. We have examined the effect of another acid, namely butyric acid, and applied this acid at relatively high concentrations. Even 8 mM butyric acid did not lead to an inhibition of Rb^+ uptake at Rb^+ concentrations

ranging from 1 to 10 mM. Neither a transient increase in cellular K^+ efflux could be detected on adding butyric acid to a suspension of metabolizing cells.

DISCUSSION

Our results are in apparent conflict with those of CONWAY AND DUGGAN[6] and of ARMSTRONG AND ROTHSTEIN[5], who found that the dependence of the Rb^+ and K^+ uptake rate by yeast upon the cation concentration obeys the Michaelis–Menten equation over the entire range of concentrations investigated. The deviation from Michaelis–Menten kinetics, however, depends upon the time of pre-incubation with glucose. The half-maximal concentration for activation is only 0.007 mM after a pre-incubation period of 11 min rising to 0.045 mM after 1 h pre-incubation. The experiments of CONWAY AND DUGGAN[6] and of ARMSTRONG AND ROTHSTEIN[5] were carried out without any pre-incubation. In addition the concentrations of Rb^+ or K^+ applied by these authors were too high to permit the detection of such small rates of activation.

It is not very probably that the activation of the Rb^+ uptake mechanism by Rb^+ and K^+ is indirect and a consequence of the stimulation of glycolysis. The maximum increase of the glycolytic rate was only 12%, whereas the Rb^+ uptake mechanism could be stimulated up to about 300%.

The mechanism of activation of the cation transport observed by us differs essentially from the mechanism of stimulation of ion uptake *via* competition of inhibiting cations like Na^+ or protons with K^+ or Rb^+ for a modifier site, as described by ARMSTRONG AND ROTHSTEIN[11]. The affinity of K^+ for this latter site is lower than the affinity of K^+ for the substrate site, a fact which is incompatible with our observation of an apparent activation constant about 40 times as small as the measured K_m.

The similarity between the results obtained by HAROLD AND BAARDA[2] for *Streptococcus faecalis* and those for yeast is striking. In both cases the uptake of Rb^+ is inhibited competitively by 2,4-dinitrophenol and the uptake of phosphate is decreased according to some kind of non-competitive inhibition. The apparent competitive inhibition of Rb^+ uptake by yeast cannot be ascribed to an increased permeability of the cells to Rb^+ giving rise to a diffusion component which would compensate the inhibition of active Rb^+ uptake more at higher than at lower Rb^+ concentrations. As a matter of fact the effect of dinitrophenol upon cell permeability is only transient, and is no longer visible after 6 min pre-incubation with dinitrophenol. Possibly the apparent competition between Rb^+ and dinitrophenol is due to an increased accessibility of the substrate site on the carrier mechanism to protons[12].

The effect of dinitrophenol upon Rb^+ release from pre-loaded metabolizing cells is different from that described by RIEMERSMA[4] for K^+ release from resting cells. The leakage of K^+ from these cells continues at least for 10 min whereas the Rb^+ outflow from metabolizing cells as shown here is stimulated for only 1 min or even shorter. The subsequent release of Rb^+ is slower than in the absence of dinitrophenol. Also ROTHSTEIN AND BRUCE[13] have observed that relatively low concentrations of dinitrophenol stimulate K^+ outflow from resting cells only and not from metabolizing cells. The occurrence of a transient stimulation of the K^- outflow was not mentioned by these authors. The mechanism by which the cells are made transiently permeable is unknown. One might hypothesize that dinitrophenol causes a temporarily depolarization of the cell membrane due to an increased conductivity of the membranes.

A decrease in the resistance of artificial phospholipid membranes is known to occur on adding dinitrophenol to the medium[14]. The subsequent permanent increase in cell permeability to Rb^+, as found almost immediately with 0.9 mM dinitrophenol or after 40 min pre-incubation with 0.28 mM dinitrophenol, resembles more the effect observed with resting cells. Apparently, metabolizing cells are able to counteract the changes in membrane structure caused initially by low concentrations of dinitrophenol.

The fact that dinitrophenol only stops Na^+ efflux (see also ref. 15), and does not give rise to a detectable stimulation of Na^+ efflux or influx, does not mean that no changes in cell permeability for Na^+ occur. The rate of active Na^+ release is about 100 times larger than the rate of Rb^+ efflux, which would mask all minor changes in passive ion movements. The inhibition of Rb^+ efflux observed with the lower dinitrophenol concentrations after the initial stimulation can be explained by assuming that the Rb^+ efflux also occurs via the same Na^+ pump though at a much slower rate than the Na^+ efflux.

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