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EFFECTS OF SODIUM AZIDE AND 2,4-DINITROPHENOL ON PHOSPHORYLATION REACTIONS AND ION FLUXES IN *SACCHAROMYCES CEREVISIAE*

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SUMMARY

NaN_3 and 2,4-dinitrophenol inhibit acid secretion by cells of *Saccharomyces cerevisiae* during glucose fermentation. Apart from ion transport, azide and 2,4-dinitrophenol also influence the course of phosphate incorporation during fermentation. A decrease of the cellular orthophosphate concentration, following glucose addition, normally occurs in two steps. The second step begins at the point where the cellular ATP concentration reaches a plateau. This plateau approximately coincides with the main phase of hydrogen-ion transport as well as with the main phase of polyphosphate synthesis. In the presence of 1 mM azide or 1 mM dinitrophenol the second stage of the decrease in orthophosphate is eliminated. The effects of azide and dinitrophenol appear to be connected with changes in cell-membrane structure.

INTRODUCTION

Active ion transport under anaerobic conditions is inhibited by 2,4-dinitrophenol in the isolated turtle bladder[1] and in cells of *Saccharomyces cerevisiae*[2]. Azide, another uncoupler of oxidative phosphorylation, has been shown to have similar effects[2]. While in respiring cells the inhibition of ion transport by uncoupling substances is readily understood, there is some difficulty in accounting for their effect on anaerobically fermenting cells. Azide and dinitrophenol interfere with respiratory energy production by preventing the formation of adenosine triphosphate in the sequence of reactions:

$$\text{respiration} \rightleftharpoons \sim(1) \rightleftharpoons \sim(2) \rightleftharpoons \sim P \rightleftharpoons ATP \quad \text{(refs. 3–5)}$$

Anaerobic energy production is not inhibited. The inhibition of ion transport by azide under anaerobic conditions could be due to interference with the ion-transport mechanism or with other routes of ATP utilization[5–8]. The present paper deals with the K^+–H^+ exchange in glucose-fermenting yeast cells and simultaneous phosphorylation reactions.

Several studies have dealt with the release of hydrogen ions by fermenting yeast cells[9–13], but few determinations of the quantity of hydrogen ions as a function of the experimental conditions have been reported. By means of a recording pH-stat

apparatus we ascertained the influence of the extracellular pH, of added potassium ions, and of various inhibitors.

If a connection exists between ion transport and energy metabolism, factors influencing ion transport could be expected to influence phosphorylation reactions, with attendant shifts in various intracellular phosphates. Time curves for acid production were correlated with time curves for certain cellular phosphorus compounds. Samples of cell suspension were taken at appropriate intervals and analyzed for orthophosphate, ATP, and inorganic polyphosphates. Contrary to previous suggestions[14,15], azide and dinitrophenol were found to influence phosphorylation reactions even under strictly anaerobic conditions.

METHODS

Materials

Fresh baker's yeast (Koningsgist, Delft) was washed by suspension in 5 volumes of distilled water, centrifugation, and resuspension. Air was bubbled through this suspension overnight to obtain cells in a reproducible 'starved' condition. The resulting suspension was centrifuged 20 min at 3000 rev./min. The packed cells were suspended in distilled water to prepare a 71 % suspension (71 g fresh yeast per 100 ml). In some experiments anaerobic conditions were obtained by adding 1 mM NaCN. In other cases N_2 was passed through the suspension overnight to obtain complete deaeration. Traces of O_2 were removed from the N_2 by a method described by MEITES[16].

NaN_3 (laboratory reagent, B.D.H.) was recrystallized from water by ethanol addition, washed with ethanol (96 %), and dried. 2,4-Dinitrophenol (puriss., Fluka) was used without further purification.

Apparatus

Recording pH-stat equipment (Radiometer Titrator-Titrigraph SBR 2/SBU 1/TTA 2) was used to follow the time-course of H^+ transport to the medium and to determine the total quantity of H^+ excreted (ΔH^+) at constant pH. A stream of purified N_2 was passed through the magnetically stirred yeast suspension in a double-walled glass vessel connected with a thermostat (30°). Further experimental details are given in the legends of the figures.

Analytical methods

Orthophosphate (P_i)

At intervals 0.5 ml was withdrawn from the fermenting yeast suspension and pipetted immediately into 4 ml ice-cool 10 % trichloroacetic acid. After standing for 15 min the suspension was centrifuged for 10 min at 3000 rev./min. 15 ml 0.2 M sodium acetate were added to the decanted supernatant. In the resulting solution phosphate was determined in the presence of labile phosphates by a colorimetric procedure as described by LOWRY AND LOPEZ[17,18], with a modification according to PEEL, FOX AND ELSDEN[19]. The residue obtained after centrifuging the trichloroacetic acid extract served as the starting material for polyphosphate determinations as described under Procedure A below.

Adenosine triphosphate (ATP)

ATP was determined in a buffered trichloroacetic acid extract, obtained as described above, by an enzymatic method[20], using phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (Boehringer).

Inorganic polyphosphates (poly-P)

Polyphosphate determinations were carried out according to two different procedures. In both procedures the quantity of long-chain polyphosphates containing 150–200 phosphate groups is estimated. Method A was more laborious and gave less reproducible results, so that Method B was preferred.

Procedure A. A sediment obtained after a trichloroacetic acid extraction of the yeast cells, followed by centrifugation (see ref. 1), was subsequently extracted by a series of 4 different solvents as described by LANGEN AND LISS[21,22]. Long-chain inorganic polyphosphates, extracted in the 4th step with 3 mM $CaCl_2$, were determined after hydrolysis in 1 M HCl for 45 min at 100°. The colorimetric procedure used was ZINZADZE[23] method; with this method no interference by Ca^{2+} was encountered.

Procedure B (see refs. 24–27). 0.5 ml yeast suspension was brought into 4.5 ml water preheated at 90° and boiled for 3–5 sec to produce complete cytolysis. The suspension was rapidly cooled with tap-water and centrifuged. The boiling-water extraction was once repeated. 2 ml 0.01 M acetate buffer (pH 4.7) and 2 ml saturated $BaCl_2$ were added to the combined supernatant liquids. After standing overnight at 4° the barium polyphosphate precipitate was separated from the supernatant by centrifugation for 10 min at 3000 rev./min. The sediment was washed with 96% ethanol by centrifugation. After hydrolysis in 1 M HCl for 45 min at 100°, phosphorus was determined by the procedure of CHEN-IO, TORIBARA AND WERNER[28].

Extracellular potassium concentration

The concentration was determined by removing, at intervals, 0.2 ml or 0.5 ml of the suspension from the fermentation vessel, and separating the cells from the medium in about 15 sec by Millipore filtration (filter HA, 0.45μ pore size). Potassium was determined in the filtrate with an Eppendorf flame photometer.

RESULTS AND DISCUSSION

The way in which the extracellular potassium concentration influences acid production from a given glucose quantity (ΔH^+) at the constant pH of 5.0 is indicated in Fig. 1 (1 mmole glucose was added). Without added KCl 420 μequiv H^+ are released to the medium. This quantity increases in the presence of 100 mM KCl to about 710 μequiv H^+. The effect of potassium ions is specific, NaCl being virtually ineffective in increasing acid production.

The effect of NaN_3, as illustrated by Fig. 1, is 2-fold. Azide (0.83 mM) decreases total acid production and also eliminates the normally found stimulation of acid production by potassium ions. Separate experiments showed that azide suppresses potassium uptake from the medium. Azide thus probably interferes with a K^+–H^+ exchange mechanism coupled with fermentation.

The acid secretion process in glucose-fermenting yeast cells is strongly influenced by the extracellular pH and potassium concentration, while being relatively independent of the internal pH and $[K^+]$ (ref. 2). Inside the cells the potassium concentration is approx. 0.17 M (see ref. 15). An outside potassium concentration of 1 mM

already stimulates H^+ transport, increasing not only the transport velocity but the total quantity of H^+ released to the medium.

2,4-Dinitrophenol suppresses the K^+–H^+ exchange while leaving fermentation intact[2,13,29]. The effective concentration is 0.1–1 mM. Iodoacetate (1 mM) suppresses fermentation as well as acid production. NaCN and acetazolamide (diamox) have no effect. When added in a concentration below the cytolytic threshold[30], cetyl pyridinium chloride and other quaternary ammonium surfactants do not diminish, but even slightly increase acid production.

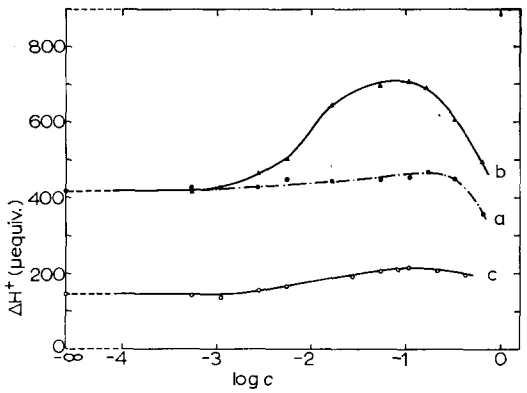


Fig. 1. Acid production as a function of extracellular $[K^+]$ and $[Na^+]$ at pH 5.0 (N_2, 30°), 15 ml 71% yeast + 3 — *a* ml water + *a* ml NaCl or KCl soln.; 0.6 ml 30% glucose added. Curve a, NaCl; Curve b, KCl; Curve c, KCl + 0.83 mM NaN_3.

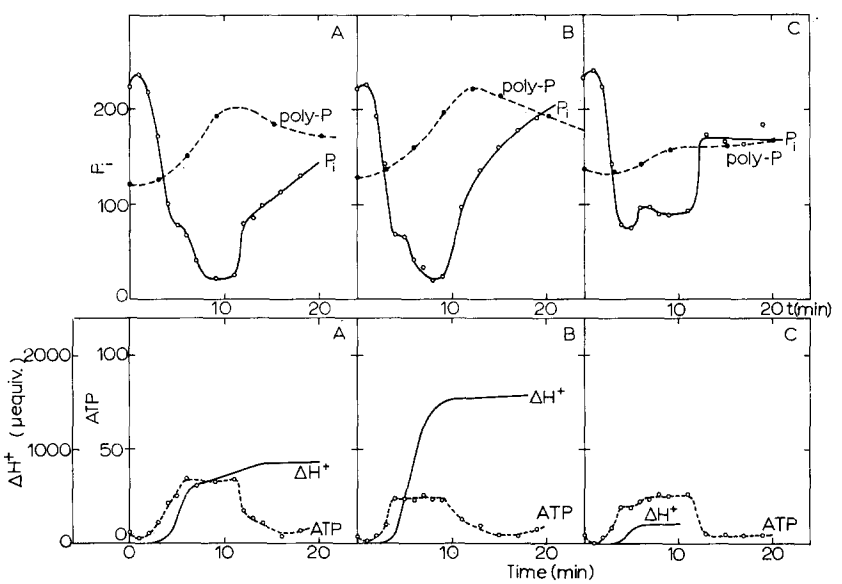


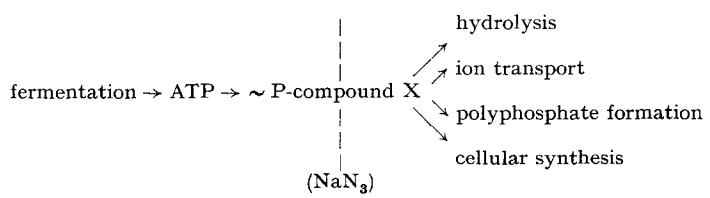
Fig. 2. Acid production and changes in orthophosphate, polyphosphate and ATP in yeast cells during fermentation at pH 5.0 under N_2, at 30°. 25 ml 71% yeast (A) + 5 ml water + 2 ml 30% glucose; (B) + 5 ml 0.6 M KCl + 2 ml 30% glucose; (C) + 4 ml water + 1 ml 31 mM NaN_3 (pH 5.0) + 2 ml 30% glucose. Glucose added at *t* is 0 min. (P_i and poly-P in μmoles P per total quantity of yeast; ATP in μmoles per total quantity of yeast.)

Earlier studies on phosphorus incorporation in yeast have shown that at the beginning of fermentation a rapid decrease in cellular orthophosphate (P_i) occurs, followed by a stationary P_i level until, at the end of fermentation, P_i shows a sudden rise[31–33]. It appears to have been overlooked that the P_i curves show an initial decrease of P_i in 2 clearly distinct stages (Fig. 2). The initial P_i fall, immediately after glucose addition, beyond a certain minimal glucose quantity, is not deepened when more glucose is added. Characteristically, the secondary fall in P_i does increase with increasing glucose quantity. While in the first stage phosphorylated compounds including ATP are formed which reach a constant level when fermentation reaches its steady state, in the secondary phase derivatory processes become important.

Fig. 2 shows how the beginning of the secondary fall in P_i coincides with the point at which the ATP concentration in the cell reaches a constant level. At approximately the same time an accelerated polyphosphate synthesis begins. H^+ transport becomes important slightly before polyphosphate synthesis. The rapid fall of P_i in the period of H^+ transport does not quite correspond to polyphosphate synthesis in this period. Especially in Fig. 2B, the case in which potassium ions are present, there is a pronounced discrepancy between P_i fall and polyphosphate increase; here polyphosphate increase continues beyond the point at which P_i reaches a minimal value.

A relation between phosphorus metabolism and H^+ transport is suggested by the fact that potassium ions have an evident influence on both processes. Azide and 2,4-dinitrophenol not only suppress H^+ transport but eliminate the secondary fall in P_i and polyphosphate formation. Physical alterations of cell-membrane structure also influence ion transport and phosphorylation. In 'active dry yeast', obtained by drying normal yeast in an air stream at 35°, structural changes have taken place which affect neither viability nor fermentation capacity but which suppress polyphosphate formation and the K^+–H^+ exchange. The P_i curves for dried and rehydrated yeast resemble those for normal yeast to which azide or dinitrophenol has been added; the secondary fall in P_i disappears (J. C. RIEMERSMA, unpublished results).

In interpreting these phenomena the energetics of glucose fermentation have to be considered. Theoretically, per mole glucose 2 moles net ATP formation are expected. The actual rise in ATP concentration represents only a small fraction of this quantity. Part of the ATP formed is used in various cellular processes, indicated in the following hypothetical scheme:



Via a postulated ~P-compound X, ATP drives various processes requiring phosphoryl transfer. Sodium azide and similar agents may facilitate the hydrolysis of ~P-compound X. An uncoupling of, for instance, ion transport and polyphosphate formation from fermentation would be the result. One would expect accordingly an increased

heat production during glucose fermentation by azide-poisoned cells. Heat production, in the presence of potassium ions which stimulate ion transport and polyphosphate formation, should be less. To verify this hypothesis, heat production during fermentation was measured under various experimental conditions.

TABLE I

HEAT EVOLVED DURING FERMENTATION

A dewar flask of 400 ml contained 200 ml 70% yeast suspension (N_2) previously brought to 30°; the solutions added were also brought to 30° before mixing. In separate experiments we added respectively (a) 40 ml water; (b) 20 ml water + 20 ml 1.2 M KCl; (c) 20 ml water + 20 ml 0.01 M NaN_3. A Beckman thermometer was used to check for temperature equilibration, and to measure the change in temperature following the addition of 8 ml 30% glucose (30°). Each value represents the mean of 4 determinations ± S.E.

Experiment	ΔT	ΔQ (*kcal per mole glucose*)
Reference suspension	1.15° ± 0.01	21.4
Suspension with 0.1 M KCl	1.05° ± 0.01	19.3
Suspension with 0.83 mM NaN_3	1.24° ± 0.01	23.2

During adiabatic fermentation in a dewar flask a temperature plateau was reached in 10–16 min, depending on experimental circumstances. This period was short enough to prevent significant heat exchange with the environment. The measured temperature increase (ΔT), together with the known heat capacity of the suspension, permitted calculation of the heat of fermentation (ΔQ) per mole glucose (Table I). Heat development under anaerobic fermentation conditions amounted normally to 21.4 kcal per mole glucose. In the presence of 0.83 mM azide this was increased to 23.2 kcal per mole glucose, whereas in the presence of 0.1 M potassium ions a decrease was found to 19.3 kcal per mole glucose.

Azide and similar reagents in 1 mM concentration appear to uncouple ion transport and inorganic polyphosphate synthesis from fermentation, while potassium ions have the opposite effect. Suppression of anabolic reactions by azide has been noted earlier, but has not been seen in relation to ion transport[34–36]. The point of attack of azide has remained obscure, and attention has been given mainly to the problem of whether this reagent suppressed net ATP formation during fermentation. Basing his argument on indirect evidence, SPIEGELMAN[37] viewed the azide effect in intact cells as primarily due to a diminished net ATP formation (see ref. 38).

The time-curves for ATP in Fig. 2 indicate that azide has a relatively minor influence on ATP formation during fermentation. Interference with ATP formation is therefore less likely than with ATP utilization. It has been demonstrated earlier that there exists in intact cells, but not in cell extracts, an azide-sensitive process which accounts for the high stationary P_i level found when azide is present[37].

Azide and 2,4-dinitrophenol interact with 'resting' yeast cells in a way suggesting a sudden change in membrane properties (see MITCHELL[39]). Endogenous metabolism was eliminated by maintaining strictly anaerobic conditions. Both agents in 1 mM concentration had an accelerating effect on the passive movements of H^+ and of K^+ through the membrane. At the moment of addition, an immediate pH

increase was found, corresponding to an inward movement of H^+. (The added solutions were freshly prepared and were brought to pH 5.0 immediately before the experiment.) While NaN_3 gave a sudden increase in pH, NaCl, acetate, and other sodium salts under similar conditions decreased the pH of the medium. The rapid pH rise due to azide was followed after 4–5 min by a more gradual rise corresponding to a slower ion leakage from the cells (see ref. 2).

Measurements of H^+ transport during fermentation, while the time of preliminary exposure of the yeast cells to azide or dinitrophenol was varied, also supported the hypothesis that these substances interact with the cell membrane. Lengthening the time of preliminary exposure from 2 to 20 min diminished the effect of the reagents mentioned. In contrast, iodoacetate had an increased inhibitory effect on H^+ production (*via* its effect on fermentation) when the time of exposure was increased from 2 to 20 min; this enzyme inhibitor diffuses to a site of action inside the cell.

Two uncouplers of respiratory-chain phosphorylation, azide and 2,4-dinitrophenol, have a pronounced and specific effect on ion transport in glucose-fermenting yeast cells. The changed course of phosphorus metabolism suggests a close relationship between a phosphorylation process coupled with the main sequence of fermentation and the translocation of ions. Both azide and 2,4-dinitrophenol may interact with a compound which is alternatively phosphorylated and dephosphorylated and which is involved in ion transport. This compound may be part of the cell membrane, as is indicated by the fact that addition of either agent to a yeast suspension results in a sudden increase in membrane permeability.

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