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THE MECHANISM OF THE PARTIAL INHIBITION OF FERMENTATION IN YEAST BY NICKEL IONS

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SUMMARY

Nickel inhibits fermentation following its transport into a non-exchangeable compartment in the cell. Extracellular pH influences the inhibition by affecting the transport. Once inside the cell, the Ni^{2+} inhibition can be modified by a number of factors such as flushing with gas, the presence of alcohol and acetaldehyde, the glucose concentration and the presence of O_2. These modifications can be explained on the assumption that the predominant effect of Ni^{2+} is to inhibit the enzyme, alcohol dehydrogenase. Changes in absorption at 345 mμ (related to the NADH level) support this suggestion. Purified alcohol dehydrogenase is highly sensitive to Ni^{2+}.

INTRODUCTION

Many heavy metal cations can inhibit the fermentation of glucose by yeast cells[1] but several different mechanisms may be involved. For example, uranyl ion inhibits by blocking the transport of sugar into the cell[2]. The inhibitory sites, in this case, are phosphoryl and carboxyl groups located on the outer surface of the cell, presumably on the plasma membrane. Virtually all of the uranyl ion taken up by the cell is bound to these sites, and it can be competitively displaced by other extracellular cations resulting in a complete reversal of the inhibitory effect. In contrast, metals reactive with sulfhydryl groups, such as mercury, silver and copper, penetrate the membrane rapidly. They not only interfere with membrane functions but react with internal sulfhydryl groups resulting in inhibition of many enzymic functions[3].

Among the metal ions that have been investigated, Ni^{2+} is of special interest because it inhibits at relatively low concentrations, but only a part of the fermentation (usually about 60%) can be blocked even at exceedingly high concentrations of the metal[1,4,5]. Because Ni^{2+} can competitively displace UO_2^{2+} from the membrane sites of yeast, it has been assumed that the two cations inhibit by the same mechanism, a blockage of sugar transport, but that Ni^{2+} produces only a partial inhibition because it introduces a smaller configurational change at the membrane sites[6].

Recently it has been demonstrated that in addition to binding to surface groups, Ni^{2+} can be transported into the cell[7]. This finding suggests that inhibitory effects of

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the metal may be due to an inhibition of glycolytic enzymes within the cell rather than of sugar transport systems on the outer surface of the membrane. In the present study, exposure of the cells to Ni^{2+} was followed by appropriate washing to remove surface-bound metal. The inhibitory effects reported are therefore related only to Ni^{2+} that was transported into a non-exchangeable pool. The predominant effect of Ni^{2+} on metabolism seems to be due to inhibition of the enzyme, alcohol dehydrogenase.

METHODS

Commercial fresh bakers' yeast (Standard Brands, Inc.) was starved under aeration for 12 h and washed three times with distilled water. The washed cells were stored at 4° and used for experiments for up to 6 days. Before each experiment they were again washed 3 times and the amount of cells was measured by a cytocrit method, using Wintrobe tubes. No correction was made for interstitial water (amounting to about 22 %) trapped between the packed cells[8].

Divalent cations taken up by yeast cells can be divided into two fractions. The first represents cations bound to anion sites on the surface of the cell. This component is exchangeable and can be rapidly displaced by adding competing cations or, in the case of radioactive isotopes, by adding non-radioactive isotopes of the same metal[9]. The second represents cations that penetrate the membrane or are transported into the cell. This component can be displaced only very slowly, if at all, by other cations or by exchange of non-radioactive isotope of the same cation[10]. Nickel binds reversibly to the surface sites[6] but the present paper is concerned only with the effect of Ni^{2+} that has been transported into the interior of the cell as a non-exchangeable component[7]. In many experiments, therefore, the cells were allowed to take up Ni^{2+} and then washed free of external Ni^{2+} before the effects on metabolism were determined.

The CO_2 and O_2 consumption were determined by standard Warburg manometry. No buffers were used because they form complexes with Ni^{2+}. Parallel systems were set up to follow the changes in pH. In non-Warburg experiments in which Ni^{2+} uptake and sugar consumption were measured, the pH was maintained at a constant level with a pH-Stat (Radiometer) using NaOH. The small amounts of Na^+ added had no effect on Ni^{2+} uptake or on metabolism.

Glucose was determined by the glucose oxidase method as modified by WASHKO AND RICE[11].

Alcohol dehydrogenase from yeast was purchased from Boehringer Mannheim Company. Enzymatic activity was determined by measuring the decrease in light absorption of NADH at 366 mμ after addition of acetaldehyde[12]. The decrease in absorption was recorded on a double-beam spectrophotometer (Unicam).

RESULTS

Nickel added to yeast under anaerobic conditions with glucose as substrate caused a partial inhibition of CO_2 production but the magnitude of this effect was dependent on the time and order of addition of nickel and of glucose (Fig. 1). The inhibition was greatest (70 %) when the cells were exposed to Ni^{2+} for 30 min before

glucose was added and minimal when the Ni^{2+} was added 5 min after the glucose. In the latter case no inhibition was evident until after 40 min. A possible contributing factor to this behavior is the change in the pH of the medium that occurs after the addition of glucose (insert of Fig. 1). The pH fell from an initial value of 5.6 to 4.3 in 30 min. The reduction in pH did not influence the rate of CO_2 production in the control (Fig. 1) but it had a remarkable effect on the inhibition by Ni^{2+} (Fig. 2).

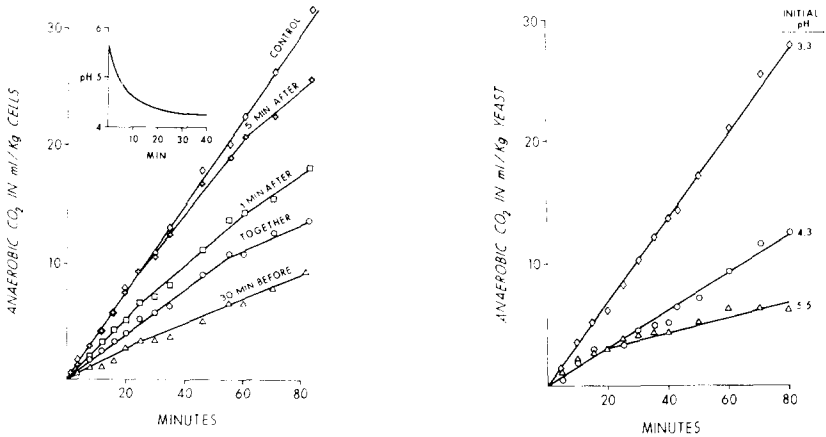

Fig. 1. Influence of the time of addition of nickel and of glucose on anaerobic CO_2 production. Inset: change in pH on addition of glucose. 5 mg yeast per flask, 5 mM glucose, 0.5 mM $NiCl_2$, gas phase N_2, temperature 28.2°.

Fig. 2. Influence of initial pH of the medium on CO_2 production in the presence of nickel. 5 mg yeast per flask, 5 mM glucose, 0.5 mM $NiCl_2$, gas phase N_2, temperature 28.2°. Initial pH adjusted to 3.3, 4.3 and 5.5 with diluted HCl.

At pH 5.5 the inhibition was considerable (compare with control in Fig. 1), but at pH 4.3, the inhibition was significantly reduced, and at pH 3.3 the rate of fermentation was virtually the same as that in a control without Ni^{2+}. A similar dependence on pH was also found by measuring the effects of Ni^{2+} on glucose utilization, measured in the same experiment.

If Ni^{2+} inhibits by blocking sugar transport at the cell membrane, then the variations demonstrated in Figs. 1 and 2 would presumably be due to differences in the amount of Ni^{2+} binding to the ligands of the cell surface. Even if the Ni^{2+} concentration was raised to 100 mM (compared to 0.5 mM used in the experiment of Fig. 2), no inhibition was evident at pH 3.3. Yet the surface groups would presumably be saturated at such high concentrations[6]. The other possible explanation is based on the pH dependence of Ni^{2+} transport, which shows marked inhibition at pH 3.3 (ref. 7). To differentiate clearly between the effects of surface-bound Ni^{2+} and transported Ni^{2+}, advantage was taken of the observation that pretreatment of yeast with phosphate *plus* glucose leads to a remarkably enhanced transport of divalent cations[10] including Ni^{2+} (ref. 7). During pretreatment the cells absorbed Ni^{2+} and they were then washed free of external Ni^{2+} before testing for inhibitory effects at different values of pH. The inhibition was about 70 % and it was not influenced by an external pH even as low as 2.5 (Fig. 3). These results are in sharp contrast to those of the experiment in which the exposure to Ni^{2+} occurred at the different pH's (Fig. 2). It

seems evident, therefore, that the inhibitory sites themselves are not accessible to extracellular pH, but that the process by which Ni^{2+} reaches the sites, the transport, is the pH-dependent process.

The first insight into the mechanism of fermentation inhibition by non-exchangeable Ni^{2+} came from an unexpected observation. Anaerobic sugar consumption of Ni^{2+}-treated cells was measured in a parallel experiment in a Warburg apparatus and in an erlenmeyer flask, the latter being used in order to take repeated samples.

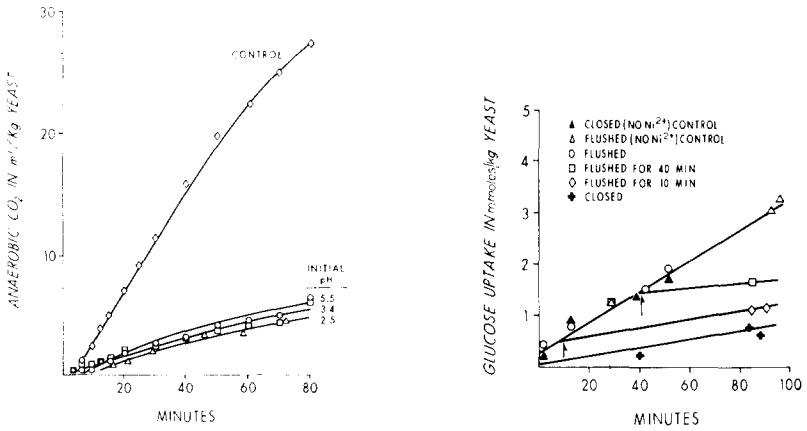

Fig. 3. The effect of pH on the CO_2 production of cells preloaded with nickel. Preloading conditions: 1% yeast, 10 mM KH_2PO_4, 0.1 mM glucose, 5 mM $NiCl_2$, 1 h, 37°. Washing procedure: 2 times distilled water, 2 times 0.1 mM succinic–tartaric acid buffer (pH 5) and again 2 times distilled water. The starting pH was adjusted with diluted HCl to 2.5, 3.4 and 5.5. 10 mg yeast per flask, 5 mM glucose, gas phase N_2, temperature 28.2°.

Fig. 4. Effect of flushing the flask with N_2 on the inhibition of glucose uptake by Ni^{2+}. Yeast, 5 mg per flask; 0.5 mM glucose, 0.5 mM $NiCl_2$, temperature 28.2°. Arrows show time at which flushing ceased and the vessels were closed.

No inhibition was observed in the erlenmeyer but the usual 60% inhibition was observed in the Warburg flask. The only difference was the means of maintaining anaerobiosis. The Warburg flask was filled with N_2 prior to the experiment and then closed, whereas the erlenmeyer flask was flushed continuously with N_2. The effect was not due to impurities in the gas because substitution of CO_2 for N_2 gave the same result. Furthermore, the same reversal of inhibition could be demonstrated by flushing with air.

An experiment demonstrating the reversal of inhibition due to flushing with gas (N_2) is shown in Fig. 4. Warburg flasks were used and sugar consumption was measured, each point representing one flask. In the absence of Ni^{2+}, the rate of sugar consumption was the same in flasks that were initially filled with N_2 and then closed just before sugar was added and in flasks in which N_2 flow was maintained continuously. In the Ni^{2+}-treated cells, however, large differences were observed. In the closed flask the inhibition was 73%, whereas in the flasks with continuous N_2 flow, no significant inhibition was observed. In flasks that were flushed for 10–40 min after the addition of glucose, the inhibition apparently started soon after the flow of N_2 was halted.

The effect of flushing cannot be attributed to differences in the uptake of Ni^{2+}

because the inhibition reappeared as soon as the flushing was terminated. It therefore seemed probable that the reversal of inhibition was due to removal of a volatile product of metabolism. Three products of fermentation are volatile: CO_2, acetaldehyde and ethanol. CO_2 seemed unlikely because the phenomenon of reversal was the same with pure N_2 or with pure CO_2. On the other hand, acetaldehyde and ethanol had large effects on the inhibition by Ni^{2+} (Fig. 5). Neither substance at the concentrations used had much effect in the absence of Ni^{2+}, but in its presence, acetaldehyde almost

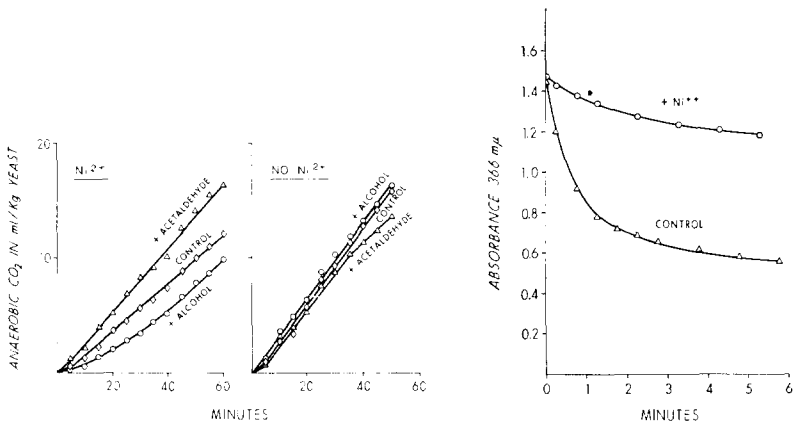

Fig. 5. Influence of alcohol or acetaldehyde on the CO_2 production of yeast cells preloaded with nickel. 5 mg yeast per flask, 5 mM glucose, 0.1% alcohol or acetaldehyde, gas phase N_2, temperature 28.2°. Preloading conditions: 0.5% yeast, 5 mM glucose, 1 mM $NiCl_2$, 1 h, 37°.

Fig. 6. Effect of Ni^{2+} on the enzymatic activity of alcohol dehydrogenase from yeast determined by the decrease in absorbance of NADH at 366 mμ. 1.5 ml 100 mM Tris (pH 8.6), 0.02 ml alcohol dehydrogenase (0.3 mg/ml), 0.08 ml NADH (20 mg/ml), 0.02 ml acetaldehyde 0.5%, 0.3 ml 5 mM $NiCl_2$, water to 3.9 ml.

reversed the inhibition, whereas alcohol intensified the inhibition. It seemed likely, therefore, that the effect of flushing with gas might be due to the continuous removal of alcohol.

The reciprocal effects of acetaldehyde and alcohol upon the Ni^{2+} inhibition suggested that the site of action was the enzyme, alcohol dehydrogenase which catalyzes the equilibrium between the two substrates. Indeed, the purified enzyme is very susceptible to inhibition by Ni^{2+} (Fig. 6). At 0.5 mM (the same concentration used in the experiments on cells), the inhibition was about 85%. Unfortunately, however, such results indicate only that the enzyme is a potential target for Ni^{2+} in the intact cell but not that it is the target or that other enzymes might not also be targets.

Further evidence for the localization of the Ni^{2+} effect was obtained by studies at different glucose concentrations under aerobic conditions. With low glucose concentrations, virtually all of the pyruvate produced by the glycolytic chain feeds into the Krebs cycle *via* pyruvate oxidase. Oxidation *via* this pathway is complete so that the R.Q. is 1.0. If, however, the glucose concentration is high, the production of pyruvate exceeds the capacity of the oxidase. It "spills over" into the pyruvate decarboxylase reaction leading to the production of "extra" CO_2 (R.Q. is above 1.0)

and of alcohol. The latter substance accumulates because oxidation *via* the two-carbon pathway cannot keep pace with production[13,14].

In view of the above properties of the yeast metabolic systems, the assumption that Ni^{2+} inhibits alcohol dehydrogenase leads to the prediction that aerobic metabolism of glucose should be insensitive to Ni^{2+}, provided the substrate concentration is sufficiently low that the R.Q. is 1.0. On the other hand, aerobic metabolism should be sensitive to Ni^{2+} only at glucose concentrations sufficiently high to produce "spillover" through the pyruvate decarboxylase and alcohol dehydrogenase (R.Q. over 1.0). Indeed, the predicted results were experimentally verified (Fig. 7). With a glucose concentration of 1 mM, the CO_2 production and O_2 consumption were equal (R.Q. = 1.0) and Ni^{2+} had no inhibitory effect. With a glucose concentration of 5 mM, CO_2 production was increased to a greater extent than the O_2 consumption (R.Q. 1.2). Both the "extra" CO_2 and "extra" O_2 were inhibited by Ni^{2+} so that the levels of CO_2 production and O_2 consumption were reduced to equality at a rate equal to that found at 1 mM glucose. Thus only the "spillover" through decarboxylase and alcohol dehydrogenase was inhibited by Ni^{2+}.

Because of the site of inhibition of Ni^{2+}, the degree of inhibition depends on the conditions and method of measurement. For example, from the data of Fig. 7, the inhibition of O_2 consumption or CO_2 production is essentially zero at 1 mM glucose, but at 5 mM glucose the inhibition of O_2 consumption is 59 %, that of CO_2 production is 67 %, and that of aerobic fermentation is virtually 100 %. On the other hand, the inhibition of anaerobic fermentation was 25 % at 1 mM glucose and 76 % at 5 mM glucose in an experiment parallel to that of Fig. 7.

Oxidation of NADH to NAD^+ is associated with the alcohol dehydrogenase reaction. The levels of NADH and of NAD^+ that are bound by the 1,3-glyceraldehyde phosphate dehydrogenase can be measured by spectrophotometric methods using extinction at 345 mμ. In yeast cells changes in the levels of NADH associated with activities of alcohol dehydrogenase can be determined[15–19]. After uptake of Ni^{2+}, the

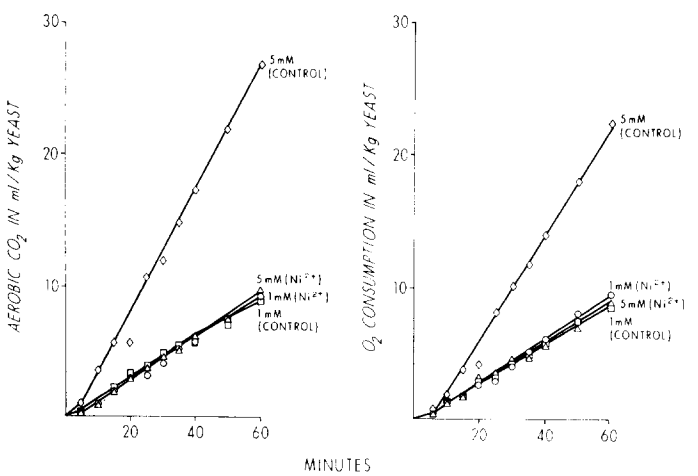

Fig. 7. CO_2 production and O_2 consumption of yeast cells loaded with nickel as in Fig. 3 at high and low glucose concentrations. 5 mg yeast per flask, 1 mM and 5 mM glucose, gas phase air, temperature 28.2°.

normal oscillations at 345 mμ upon addition of glucose are eliminated and the rise in extinction is delayed. The final value of the extinction at 345 mμ is considerably higher than in the control suggesting an elevated level of NADH. These results support the suggestion that Ni^{2+} inhibits at the level of alcohol dehydrogenase, reducing the rate of oxidation of NADH.

DISCUSSION

The inhibitory effects of Ni^{2+} reported in this paper concern transport of the metal into the cell[7] rather than its binding to surface anions[6]. Thus the effects are observed in cells preloaded with Ni^{2+} after the surface-bound Ni^{2+} has been washed away. In such preloaded cells, the inhibition is independent of extracellular pH but it is dependent on other factors such as flushing with gas (N_2, CO_2, or air), the addition of alcohol and acetaldehyde, the presence of O_2, and the concentration of glucose. The pattern can be readily understood on the basis that Ni^{2+} inhibits the enzyme, alcohol dehydrogenase, thereby reducing the supply of NAD^+, essential for the phosphoglyceraldehyde dehydrogenase reaction. Flushing would remove alcohol, thereby allowing the reaction acetaldehyde-to-alcohol to proceed more rapidly. This would increase the supply of NAD^+ and increase the rate of fermentation. The same effect is achieved by the addition of acetaldehyde. Addition of alcohol, on the other hand, would back up the reaction, reduce the production of NAD^+, and increase the inhibition.

In the presence of O_2, alcohol dehydrogenase is not involved in sugar metabolism provided the concentration of substrate is low. As expected, Ni^{2+} has no inhibitory effect. When the sugar concentration is raised to a higher level so that "spillover" through the decarboxylase reaction occurs along with the formation of alcohol[13,14], then Ni^{2+} inhibits the extra metabolism associated with the "spillover". These experiments indicate that none of the glycolytic reactions between glucose and pyruvate and none of the decarboxylic acid cycle and respiratory reactions are inhibited by Ni^{2+}. They do point to the alcoholic dehydrogenase as the most likely site. Changes in the level of NADH in cells loaded with Ni^{2+} also are consistent with this mechanism. Finally, purified alcohol dehydrogenase is very sensitive to Ni^{2+}.

Ni^{2+} has been of considerable interest among the metal ions because, even in high concentrations, it only partially inhibits glucose metabolism[1] and glucose uptake[5,6]. An explanation has been presented in terms of configurational changes in the binding groups (phosphoryl groups) of the outer surface of the cell membrane[6]. On the basis of the present study, another explanation is given based on transport into the cell and subsequent inhibition of the enzyme, alcohol dehydrogenase. Even the complete inhibition of this enzyme would allow CO_2 production and glucose uptake to proceed at a reduced rate *via* the third mode of fermentation[20] with NAD^+ produced by the conversion of dihydroxyacetone phosphate to α-glycerophosphate by the enzyme glycerophosphate dehydrogenase with glycerol as one of the final end products.

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