Mode of Action of Netzahualcoyone

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The activity of nettahualcoyone on Bacillus subtilis and Escherichia coli was studied. The product inhibited the respiration of intact cells of B. subtilis but had no effect on the respiration of E. coli. However, when preparations of sonically disrupted cells were examined, inhibitory activity on both bacteria was observed.

Netzahualcoyone is a triterpenquinone isolated from some members of the plant family Celastraceae (2). It has shown broad-spectrum antibiotic activity against gram-positive bacteria and yeasts but not against gram-negative bacteria (3). Its MIC (1.5 to 1.6 μg/ml) for Staphylococcus aureus can be compared favorably with that reported for other antibiotics, while its MIC for Bacillus subtilis is 1.25 to 0.625 μg/ml, and for Escherichia coli it is more than 200 μg/ml. The drug possesses both the triterpenquinone skeleton and the ketone group at C-22 in ring E needed to optimize antimicrobial activity (4).

This substance’s mechanism of action against microorganisms is not known, and this article gives an account of how nettahualcoyone affects the respiration of Saccharomyces cerevisiae and bacteria.

Netzahualcoyone was extracted from the roots of Schaefferia cuneifolia Stanley collected in northwest Mexico (2). As we did not know the conditions for gas chromatography or high-pressure liquid chromatography analysis of nettahualcoyone, its purity was checked by 1H nuclear magnetic resonance spectra (0.1 μM, CDCl3). Other products were not detected. B. subtilis CECT 39, E. coli CECT 99, and S. cerevisiae X 2180A were used. Unless otherwise stated, the bacteria were cultured and maintained on nutrient agar (Oxoid) at 37°C. S. cerevisiae was maintained on Sabouraud agar (Materiales y Reactivos, S. A.).

A medium containing 1% yeast extract, 2% peptone, and either 2% glucose or 2% ethanol was inoculated with S. cerevisiae, and nettahualcoyone, previously dissolved in pure dimethyl sulfoxide, was added until a final concentration of 30 or 60 μg/ml was reached. The final concentration of dimethyl sulfoxide in the cultures was 1% (vol/vol). S. cerevisiae was grown under aeration at 28°C. Growth was monitored by measuring the OD630 and by counting viable cells on Sabouraud agar plates (Fig. 1). Cultures without nettahualcoyone but in medium containing a similar volume of dimethyl sulfoxide were used as controls.

Netzahualcoyone (30 μg/ml) inhibited the growth of S. cerevisiae in medium with ethanol as a carbon source. When the carbon source was glucose, no growth inhibition was observed at 30 μg/ml, and the only visible effect was a prolongation of the lag phase when the drug was present at 60 μg/ml. As nettahualcoyone was still present after 20 h of incubation, the growth observed in medium containing glucose and nettahualcoyone at 60 μg/ml indicated tolerance to the product. The difference between the effects on yeast growth on glucose and on a nonfermentable substrate suggested cell respiration as a possible target process.

In order to study the effect of bacterial respiration, bacteria were suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 1% glucose to a cell density of 10^7/ml.

FIG. 1. Growth curves of S. cerevisiae on glucose (A) or ethanol (B) as a carbon source. Symbols: ▲, controls without nettahualcoyone; ○, cultures with nettahualcoyone at 30 μg/ml; ●, cultures with nettahualcoyone at 60 μg/ml.

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Oxygen consumption was measured at room temperature first without and then with netzahualcoyone (7 or 14 μg/ml) with a Clark oxygen electrode (Fig. 2A and B). The effect of oxygen uptake by *B. subtilis* was dependent on the cell density and was greater at cell densities of 10⁵/ml or less. Under the same conditions, *E. coli* was unaffected, as were the other bacteria tested. Similar results were obtained when the cells were suspended in different media. These results indicated that netzahualcoyone had an inhibitory effect on the cellular respiration of the microorganisms and were in line with previous findings of its effect on the growth of *S. cerevisiae* and its MICs for *B. subtilis* and *E. coli*.

Disrupted cell preparations were made as follows. Cells were grown in YEPD medium at 37°C under aeration and collected by centrifugation. The following procedures were all carried out at 5°C. The cells were washed twice in 0.1 M potassium phosphate buffer (pH 7.0), suspended in the same buffer in the ratio of 1 g (wet weight) of cells to 5 ml of buffer, and broken sonically (Sonifier B 12; Branson Sonic Power Co.), in 30 10-s bursts with 20-s intervals (15 min in all). The efficiency of lysis was checked by monitoring the release of UV-absorbing material, which was maximal after the described treatment. Intact cells were removed by centrifugation at 4,000 x g for 15 min. The supernatant was then centrifuged at 18,000 x g for 15 min, and the pellet was resuspended in the same buffer as before (5 ml). Aliquots (0.1 ml) were incubated at room temperature in the same buffer (2.8 ml) containing NADH (0.1 mM). The oxidation of

FIG. 2. Oxygen uptake by cell suspensions of *B. subtilis* (A) and *E. coli* (B) at 10⁷/ml in 0.1 M potassium phosphate buffer containing 1% glucose. At the times indicated by the arrows, netzahualcoyone (7 or 14 μg/ml) was added to the suspensions. (C) Oxygen uptake by *B. subtilis* disrupted cell preparations suspended in 0.1 M potassium phosphate buffer containing 0.1 mM NADH as a substrate for respiration. Netzahualcoyone (40 μg/ml) was added at the time indicated by the arrow. ———, Without drug; ———, with drug.

FIG. 3. NADH (0.1 mM) oxidation by *E. coli* (A and B) and *B. subtilis* (C) disrupted cell preparations. (A and C) Netzahualcoyone (10 μg/ml) was added at the time shown by the arrow. ———, Without drug; ———, with drug. (B) 10 mM sodium dithionite (———) or 6 mM KCN (———) was added at the time shown by the arrow. ———, No addition.
NADH was measured by reading the A_{340} with and without netzahualcoyone (10 μg/ml). KCN (6 mM) and sodium dithionite (10 mM) were used as controls (Fig. 3). As KCN at this concentration is a powerful alkali, it was neutralized just prior to addition.

Membrane preparations from both B. subtilis and E. coli oxidized NADH, as can be seen from the drop in A_{340}. In both cases, when netzahualcoyone (10 μg/ml) was added, the slope of the graph leveled off, representing an almost sevenfold reduction in the oxidation rate of the substrate. When netzahualcoyone was not present, NADH oxidation depended on the amount of oxygen dissolved and on respiratory chain function, as evidenced by the fact that both sodium dithionite (10 mM) and KCN (6 mM) inhibited the process. A control experiment eliminated the possibility that sodium dithionite reduces NAD\textsuperscript{+} directly to NADH.

Spontaneous NADH oxidation or reduction of NAD\textsuperscript{+} in the presence of netzahualcoyone was not observed, which suggests that the inhibition recorded was a consequence of the inhibition of respiratory chain functions, which agrees with the reduction in oxygen uptake by disrupted cells of B. subtilis (Fig. 2C).

The effects of netzahualcoyone on the cellular respiration of intact E. coli cells and disrupted cell preparations are particularly interesting because they suggest that cell membranes of both gram-positive and gram-negative bacteria are sensitive to its action. Therefore, the insensitivity of the intact gram-negative bacteria may be due to the existence of a permeability barrier, probably the outer membrane of these bacteria.

To study the possible effect on cell permeability, the experimental procedure of Chou and Pogell (1) was adopted. Cells in log phase were washed with 0.1 M potassium phosphate buffer (pH 7.0) and then suspended in the same buffer at an OD\textsubscript{620} of 0.62. The suspension was divided into two equal parts, and netzahualcoyone (10 μg/ml) was added to one. Both samples were incubated at 37°C under aeration. The optical density of the suspensions was determined at intervals, and after the cells had been removed by centrifugation in a Microfuge (Beckman), the absorbance of the supernatant at 260, 280, and 460 nm (the maximal absorption for netzahualcoyone) was determined (Fig. 4A and B).

The effect of netzahualcoyone on UV-absorbing material leakage (A\textsubscript{260} and A\textsubscript{280}) and the integrity of B. subtilis was measured (A\textsubscript{620}) for 120 min. The A\textsubscript{620} was constant for preparations both with and without netzahualcoyone (data not shown), suggesting that the cells did not undergo lysis during the incubation time. The A\textsubscript{260} and A\textsubscript{280} of the supernatant indicated that when netzahualcoyone was present, there was always some decrease in UV-absorbing material leakage, and this suggests that in presence of netzahualcoyone, there is less cell lysis, perhaps because of inhibition of autolytic enzymes.

The following facts favor the idea that netzahualcoyone is adsorbed on the bacterial cell surface: during incubation, the color of the cell sediments turned progressively darker, and the maximal A\textsubscript{340} for netzahualcoyone in the supernatant was lower each time it was measured, indicating that the product was adsorbed to or penetrated the cells.

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