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Physiologic actions of zinc related to inhibition of acid and alkali production by oral streptococci in suspensions and biofilms

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Zinc is a known inhibitor of acid production by mutans streptococci. Our primary objective was to extend current knowledge of the physiologic bases for this inhibition and also for zinc inhibition of alkali production by *Streptococcus rattus* FA-1 and *Streptococcus salivarius* ATCC 13419. Zinc at concentrations as low as 0.01–0.1 mM not only inhibited acid production by cells of *Streptococcus mutans* GS-5 in suspensions or in biofilms but also sensitized glycolysis by intact cells to acidification. Zinc reversibly inhibited the F-ATPase of permeabilized cells of *S. mutans* with a 50% inhibitory concentration of about 1 mM for cells in suspensions. Zinc reversibly inhibited the phosphoenolpyruvate: sugar phosphotransferase system with 50% inhibition at about 0.3 mM $ZnSO_4$, or about half that concentration when the zinc–citrate chelate was used. The reversibility of these inhibitory actions of zinc correlates with findings that it is mainly bacteriostatic rather than bactericidal. Zinc inhibited alkali production from arginine or urea and was a potent enzyme inhibitor for arginine deiminase of *S. rattus* FA-1 and for urease of *S. salivarius*. In addition, zinc citrate at high levels of 10–20 mM was weakly bactericidal.

Key words: alkali production; glycolysis; oral streptococci; zinc

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Zinc is added as an antimicrobial agent to many oral healthcare products, notably toothpastes. It is commonly added along with citrate, which has anti-calculus action. The antimicrobial actions of zinc citrate appear to be due mainly to the zinc component. Zn^{2+} is well known as a sulfhydryl reactive agent, and many of its antimicrobial actions may be caused by reaction with sulfhydryl groups (8, 22). For example, zinc inhibition of the trypsin-like protease of *Porphyromonas gingivalis* was found (18) to be reversed by the sulfhydryl compound dithiothreitol. Chelators such as citrate or ethylenediaminetetraacetate may lower the antimicrobial potency of zinc (28). However, the results of many studies have shown that zinc citrate is an active antimicrobial *in vitro* and a substantive antiplaque agent in the mouth (7). The complex ionic interactions in dental plaque may allow for zinc removal from chelate complexes because of the high affinities of oral streptococci for the mineral (25). Moreover, many bacteria can take up heavy metals more readily from chelates than from free ionic forms.

Zinc has been shown to be an inhibitor of acid production by human dental plaque *in vitro* (14) and *in vivo* (21). Watson et al. (28) found that 0.1 mM Zn^{2+} added as the sulfate salt caused 50% inhibition of acid production from glucose by *Streptococcus mutans* under pH-stat conditions at a pH of 7. There was less inhibition at pH 6 and almost no inhibition at pH 5. Zinc is generally considered to be only bacteriostatic (8). However, Izaguirre-Fernández et al. (16) did find that Zn^{2+} can be bactericidal for *S. mutans* in the presence of fluoride at low pH values. Also, zinc can enhance the bactericidal action of agents such as triclosan (8). He et al. (15) have recently extended previous studies by demonstrating that zinc inhibits the glycolysis of many organisms from supragingival plaque at concentrations as low as 0.01 mM. In their studies, inhibition was not greatly affected by pH changes in the range from 7.0 to 5.0. Moreover, the inhibitory potency of Zn^{2+} was reduced when packed cell layers were used instead of cells in suspensions. Giertsen et al. (12) showed that alkali production from urea by dental

plaque was also inhibited by zinc. Wunder & Bowen (29) found that glucosyltransferases of *S. mutans* were inhibited by zinc when the enzymes were in solution but not bound to surfaces. Overall, it is clear that zinc is an effective antimicrobial agent against mutans streptococci and a variety of oral bacteria with multiple inhibitory actions, including actions on exoenzymes, as reviewed by Cummins (8) and by Marsh & Bradshaw (19).

Despite the information available in the literature on the antimicrobial actions of zinc against oral bacteria, there are still important questions to be answered. For example, is zinc effective against cells in mono-organism biofilms? Does it affect acid tolerance as well as acidogenesis? Can it be bactericidal or only bacteriostatic? Our major aim in the work described here was to explore in more depth the physiology of zinc inhibition of acid and alkali production by oral streptococci.

Materials and methods

Bacteria

S. mutans GS-5, *Streptococcus rattus* FA-1 and *Streptococcus salivarius* ATCC13419 are maintained routinely in our laboratory with weekly subculture on tryptic-soy agar plates (Difco, Detroit, MI) to avoid selecting rapidly growing variants. Long-term storage is at –70°C in 50% glycerol solution. For work with cell suspensions, bacteria were grown in static cultures in a standard 37°C incubator in tryptone-yeast-extract (TY) medium (1). For most of the work, the medium contained 1% (w/v) glucose (55.6 mM), so that growth was acid-limited rather than catabolite-limited. However, for induction–derepression of the arginine deiminase system of *S. rattus*, the TY medium was supplemented with only 0.1% glucose and with 1% arginine (47.5 mM).

Mono-organism biofilms were grown on glass slides following procedures described previously (5, 24). The slides were placed initially in TY Sucrose medium in glass staining dishes and transferred to new medium daily until dense biofilms formed, usually some 3×10^8 cells/mm^2 on slides with a total area of 18.75 cm^2. The day before the biofilms were to be used for experiments, generally 4–7 days after inoculation, they were transferred overnight to the final medium, such as TYGlucose or TY with a low level of glucose (0.1%) and a high level (1.0%) of arginine. This final growth allowed for induction–derepression of catabolic systems such as the arginine deiminase system. Biofilms were harvested by removing them from the final growth medium, washing them with salt solution (50 mM KCl plus 1 mM $MgCl_2$) and then using them directly for experiments. They could be dispersed by scraping them from the glass into salt solution, homogenizing with a tissue homogenizer and then briefly sonicating, as described previously (5). The procedure does not kill oral streptococci but does lead to complete dispersal.

Cells were permeabilized as described previously (1) by treating them with 10% (vol/vol) toluene and then subjecting them to 2 cycles of freezing and thawing.

Glycolysis assays

Initial evaluations of the effects of zinc on glycolysis involved standard pH-drop experiments with thick cell suspensions or biofilms of the sort we have described previously (2). In subsequent experiments, assays of glycolysis at constant pH values were performed. Cells from suspension cultures or intact biofilms were harvested, washed once with salt solution (50 mM KCl plus 1 mM $MgCl_2$), and resuspended in salt solution. The pH was adjusted to ca. 7.2 with KOH solution, sufficient sugar was added to give a concentration of 1% (w/v), and the fall in pH was assessed with a glass electrode. For pH-drop experiments with intact biofilms, the cells remained attached to the glass slides on which the films were formed.

Glycolysis was assayed in two ways, in addition to recording pH drop, either by determining production of titratable acid or by measuring disappearance of glucose. Acid production was assessed under pH-stat conditions from recordings of the amount of standardized KOH solution required to maintain the pH at a set value. Acid production was expressed in terms of μequivalents/mg cell dry weight. When biofilms were used, an entire biofilm on its slide was placed in salt solution in a tube, pH was adjusted to the desired value, substrate added, and acid production assayed by titration with base. Dry weights of biofilms were determined by scraping them from the glass sides into water, centrifugation of the cells and washing again with water. Samples of the final water suspensions were dried to constant dryness in an oven. Disappearance of glucose was assayed using standard glucose-oxidase reagents from Sigma Chemical Co. (St. Louis, MO).

Alkali production was assayed in terms of pH rise with the same procedures used to assess pH drop, except that the starting pH value was adjusted to about 5.

Enzyme assays

F-ATPase activity was determined with permeabilized cells in terms of phosphate release from ATP assayed as described previously (1). Phosphate was assayed chemically, as described previously (27). Phosphotransferase system (PTS) activity was assessed with permeabilized cells in terms of pyruvate production from phosphoenolpyruvate in response to glucose or sucrose addition as described previously (3). Pyruvate was assayed by use of lactic dehydrogenase and measurements of the change in absorbance of 340-nm light associated with oxidation of NADH. Pyruvate kinase was assayed in terms of pyruvate formation from phosphoenolpyruvate. The reaction mix contained 50 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$, 100 mM KCl, 0.5 mM EDTA, 10 mM ADP and permeabilized cells. The reaction was initiated with 10 mM phosphoenolpyruvate. Again, pyruvate was assayed with lactic dehydrogenase, which was found to be insensitive to inhibition by zinc at the levels used to inhibit the PTS or pyruvate kinase.

Permeabilized cells were used also for measurement of arginine deiminase enzyme activity in terms of production from added arginine of citrulline, which was assayed chemically as described by Curran et al. (9). Urease activity was also assessed in terms of ammonia production from urea by permeabilized cells. NH_3 was assayed by use of ammonia assay kits from Sigma Chemical Co. (St. Louis, MO) based on the reaction catalyzed by glutamate dehydrogenase.

Levels of glycolytic intermediates within cells were determined enzymatically as described previously (3) using cells starved to reduce intermediate levels, and control cells given excess glucose and allowed to come to steady-state glycolysis and not exposed to $ZnSO_4$, or cells in steady state glycolysis treated with 0.10, 0.25 or 1.00 mM $ZnSO_4$, all at pH 7. The intermediates were extracted from cells with perchloric acid.

Killing assays

Killing assays for cells in suspensions and in biofilms were basically those described by Ma et al. (17). Samples of cell suspensions treated with zinc salts or biofilms treated with zinc and then dispersed were transferred to tubes containing 1% (w/v) Difco peptone broth. The suspensions were mixed and used for further dilution or for plating on trypticase-soy agar (Difco). The plates were then incubated at 37°C aerobically for 24–48 h until no more colonies appeared. Peptone broth was used as diluent

Table 1. Effects of zinc sulfate on intracellular levels of glycolytic intermediates in cells of *S. mutans* GS-5 at pH 7.0

	% value for untreated, glycolyzing cells[1]				
Compound	Starved cells	Glycolyzing cells (GC)	GC ± 0.1 mM $ZnSO_4$	GC ± 0.25 mM $ZnSO_4$	GC ± 1.0 mM $ZnSO_4$
G6P	1.3 ± 1.8[a]	100 ± 0	72.3 ± 15.3	70.7 ± 12.0	*45.0 ± 8.8*[2]
F6P	11.3 ± 3.4	100 ± 0	83.3 ± 5.3	90.7 ± 15.3	96.3 ± 45.0
FBP	21.0 ± 20.8	100 ± 0	123.2 ± 47.0	87.0 ± 43.8	67.0 ± 12.2
DHAP	43.7 ± 7.0	100 ± 0	110.1 ± 34.2	95.3±47.8	104.2 ± 25.9
GA3P	35.4 ± 24.5	100 ± 0	74.3 ± 18.7	54.2 ± 16.5	61.3 ± 7.5
3PGA	127.4 ± 62.7[2]	100 ± 0	36.1 ± 27.2	11.2 ± 15.6	**0.0 ± 0.0**[b]
2PGA	199.7 ± 1.90	100 ± 0	81.2 ± 26.9	52.2 ± 55.1	**8.3 ± 11.8**
PEP	190.0 ± 103.4	100 ± 0	135.7 ± 18.1	235.4 ± 45.5	55.3 ± 39.5
PYR	20.0 ± 11.6	100 ± 0	90.7 ± 11.3	63.3 ± 9.5	**10.2 ± 3.5**
LACT	11.7 ± 6.2	100 ± 0	92.3 ± 3.2	80.3 ± 2.1	71.4 ± 7.1

[1]Mean ± standard deviation, $n = 3$. For each of the three experiments, the value for the control suspension of glycolyzing cells not treated with zinc was set at 100%. Then the values for the zinc-treated or starved cell suspensions were expressed as percentage of the control value.
100% values (μmol/g cell dry weight) are: G6P-12.5; F6P-2.91; FBP - 12.27: DHAP - 1.66; GA3P-1.76; 3PGA - 2.72; 2PGA - 3.26; PEP - 2.35; PYR - 54.9; LACT - 1055.4.
[2]Entries in italics indicate more than 50% reduction from the control value. Entries in bold indicate 90 or more % reduction from control values. Underlined entries indicate increases over control values of more than 25%.

not only to lower the zinc concentration but also to allow for zinc binding by peptone ingredients to stop killing.

Proton permeability determinations

The permeabilities of cells to protons were assessed as described previously (24). Basically, cells were harvested and washed with salt solution. They were then starved briefly with pH adjustment until a desired steady pH value was achieved. Then, HCl was added to drop the pH value by about 0.2 units. The subsequent rise in pH associated with movement of protons across the cell membrane into the cytoplasm was recorded with a glass electrode, which can sense protons outside the cell but not those within the high-capacitance cell membrane. After an interval of recording, butanol was added to disrupt cell membranes. A rapid rise in pH followed, indicative of loss of ΔpH between the cytoplasm and the environment.

All experiments were repeated at least twice, and three or more repeats allowed for calculation of standard deviations indicated by error bars in the figures and entries in Table 1.

Results

Inhibition of glycolysis

Typical results concerning the effects of Zn^{2+} on pH drop in suspensions of *S. mutans* GS-5 with excess glucose are presented in Fig. 1(A). Rates of pH drop reflect acidogenic capacities of the cells, while final pH values of the suspensions also reflect acid tolerance. For acid-tolerant mutans streptococci, the final pH values are typically slightly below 4, as shown here for the GS-5 strain. When 0.010 mM Zn^{2+} was added, either as the sulfate or chloride salt, it had only a small effect on the rate and extent of acidification. The addition of 0.015 mM Zn^{2+} significantly slowed glycolysis, and 0.025 mM nearly completely stopped acid production from glucose.

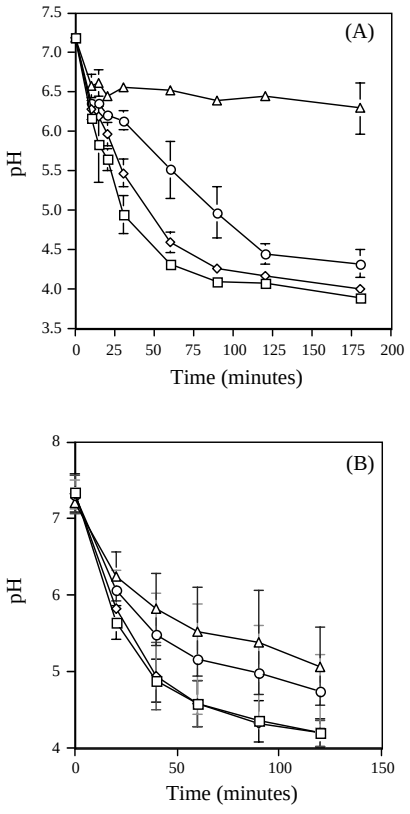

Fig. 1. Effects of Zn^{2+} on glycolytic pH drop by cells in suspensions (A–0.4 mg cell dry weight/ml) or in biofilms (B – 171 mg cell dry weight per slide area of 18.75 cm^2 in a suspending volume of 45 ml) given excess glucose just after initial pH adjustment. Error bars indicate standard deviations with $n =$ at least 3. pH was measured with a glass electrode in the suspending medium. Symbols in panel A: -□- control, -◇- 0.010 mM Zn^{2+}, -○- 0.015 mM Zn^{2+} and -△- 0.025 mM Zn^{2+}. Symbols in panel B: -□- control, -◇- 0.02 mM Zn^{2+}, -○- 0.10 mM Zn^{2+} and -△- 0.20 mM Zn^{2+}.

Zn^{2+} not only slowed glycolysis but also affected the final pH value. In other words, it affected both acidogenesis and acid tolerance. The effects were reversible when cells were washed with salt solutions and used for new pH-drop assays (data not shown). Zinc citrate induced a somewhat more potent inhibition than sulfate or chloride salts. Biofilms were less sensitive to Zn^{2+} inhibition (Fig. 1B) than were cells from suspension cultures in dense suspensions. However, glycolysis and acid production by biofilms could be inhibited by zinc at levels of some 4–20 times those required for similar effects on cells in suspensions.

Zinc inhibited acid production from glucose by *S. mutans* GS-5 in cell suspensions under pH-stat conditions (Fig. 2), here at a pH value of 7. Inhibition was clearly

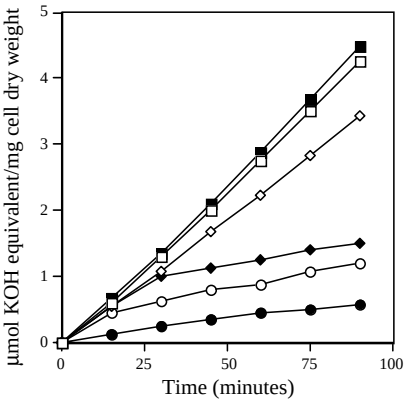

Fig. 2. Zinc inhibition of acid production by cells of *S. mutans* GS-5 in suspensions with 1.1 or 4.4 mg cell dry weight per ml at a constant pH of 7. -□- control at cell density of 4.4 mg dry weight/ml, -■- control at cell density 1.1 mg cell dry weight/ml, -◇- 0.025 mM Zn^{2+} at cell density 4.4 mg/ml, -◆- 0.025 mM Zn^{2+} at cell density 1.1 mg/ml, -○- 0.100 mM Zn^{2+} at cell density of 4.4 mg dry weight/ml and -●- 0.100 mM Zn^{2+} at cell density of 1.1 mg/ml.

affected by the cell density of the suspensions used. Under the experimental conditions, cell density had little effect on control rates of acid production per mg cell dry weight. However, cells in less dense suspensions (1.1 mg cell dry weight per ml) were clearly more inhibited by Zn^{2+} at the concentrations used than were cells in more dense suspensions (4.4 mg cell dry weight per ml). Thus, zinc action is similar to that of many antimicrobial agents for which there appear to be binding sites on cells effective for inhibition as well as those ineffective. In fact, there was a linear, inverse relationship between potency, say, 50% inhibitory concentration, here for zinc, and biomass concentration in suspensions. The lower sensitivity to zinc of biofilms, compared with cells in suspensions (Fig. 1) appears to be related largely to the high cell densities of the films. Also, glycolysis by biofilms was slower than that by cells in suspensions, probably mainly because of diffusion limitations in the biofilms.

When the pH value for suspensions or biofilms was reduced to 5.0, glycolysis was still inhibited, but higher concentrations of Zn^{2+} were required. For example, the 50% inhibitory concentration for cells in suspensions increased from about 0.02 mM at pH 7 to about 0.20 mM at pH 5. When glycolysis was assayed in terms of glucose disappearance, essentially the same results were obtained (data not shown). Also, we routinely assessed effects of zinc citrate because it is the commonly used zinc salt in oral care products. In general, zinc citrate was more effective than the sulfate salt. For example, for inhibition of glycolysis by cells in suspensions at pH 5, the 50% inhibitory concentration for zinc citrate was about half that of zinc sulfate.

Inhibition of F-ATPase and increased proton permeability

Since zinc reduces acid tolerance of *S. mutans* as indicated by the final pH values in pH-drop experiments, it may inhibit the F-ATPase, which is the major engine of acid tolerance in this organism. As shown in Fig. 3(A), the F-ATPase activity of permeabilized cells of *S. mutans* GS-5 was reversibly inhibited by Zn^{2+} with a 50% inhibitory concentration of about 1 mM. When one compares the pH-drop data in Fig. 1(A) to the F-ATPase sensitivities to zinc in Fig. 3(A), the changes in acid tolerance, reflected by changes in final pH values, are greater than might be expected from F-ATPase inhibition. However, zinc acts to slow glycolysis even at higher pH values (Fig. 2) as well as inhibiting the F-ATPase and sensitizing glycolysis to acidification. The results of previous work (2) indicate that the rate at which ATP can be supplied from catabolism affects acid tolerance. Basically, higher rates of supply result in greater acid tolerance. Therefore, zinc has dual actions leading in reduced acid tolerance – inhibition of ATP synthesis and inhibition of F-ATPase. The other factor to be considered in comparing the two sets of results is that the F-ATPase assays were carried out with cell suspensions of about twice the biomass concentration used for pH-drop experiments, and the greater biomass would somewhat decrease the inhibitory potency of zinc.

Inhibition of the F-ATPase would be expected to increase the permeability of cells to protons. This effect is reflected by the data presented in Fig. 3(B). In this type of assay, cells in suspensions were initially equilibrated at a steady pH value of about 4.7. Then, HCl was added to drop the pH rapidly to the value indicated. The rise of pH of a suspension as protons moved into the cell was recorded with a glass pH electrode. When protons move across the cell membrane, they no longer can be detected by an electrode outside the cell. The effects of zinc were not as great as might be expected from the sensitivity of the F-ATPase of permeabilized cells. However, the proton permeability assays are, of necessity, carried out with intact rather than permeabilized cells. Still, in Fig. 3(B) it is apparent that the dynamic permeability to protons was increased and, moreover, that the pH rises after butanol addition indicated that exposure, especially to 0.25 mM Zn^{2+}, also diminish the ΔpH across the cell membrane. Zinc citrate could not be tested easily in this type of experiment because citrate is a buffer ($pK_{a2} = 4.76$; $pK_{a3} = 6.40$.) in the pH range studied.

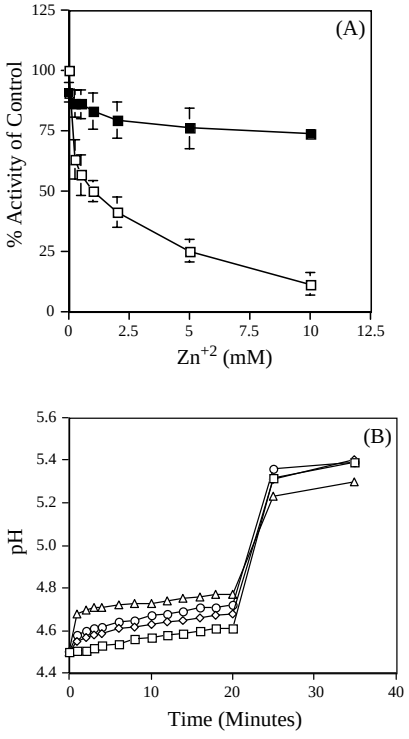

Fig. 3. (A) Zinc inhibition of F-ATPase activity of permeabilized cells of *S. mutans* GS-5 in suspensions (0.8 mg cell dry weight per ml based on the original weight of the cells permeabilized) either washed (-■-) or not washed (-□-) after zinc sulfate treatment. Error bars indicate standard deviations with $n = 3$. The open symbols indicate activity when zinc remained in the suspension, while the closed symbols indicate activity when the cells were washed to remove zinc. 100% activity = 0.064 μmol Pi/mg cell dry weight/min. (B) Effects of zinc on the proton permeabilities of cells of *S. mutans* GS-5 in suspensions at a cell density of 0.4 mg dry weight per ml. At 20 min, butanol was added to the suspensions to damage the cell membrane. -□- control, -◇- 0.050 mM Zn^{2+}, -○- 0.100 mM Zn^{2+} and -△- 0.250 mM Zn^{2+}.

Inhibition of phosphoenolpyruvate: sugar phosphotransferase system

As reported previously in the literature (8), the PTS of oral streptococci is sensitive to the inhibitory effects of zinc. As shown by the data presented in Fig. 4(A), the 50% inhibitory concentration in our experiments was close to 0.3 mM. Inhibition was mainly reversible so that full inhibition occurred only when zinc remained in the assay mix. Again, the reversible type of action is reasonable in terms of the bacteriostatic rather than bactericidal effects of zinc on bacteria. The PTS can be inhibited readily by zinc citrate as well as by zinc added in the form of the dissociable sulfate salt. Again, zinc citrate was somewhat more effective, as shown by the dose-response curves in Fig. 4(B). The exact nature of zinc inhibition of the PTS remains to be determined. It may be related simply to zinc cations competing with cations such as Mg^{2+} required for PTS activity. However, zinc inhibition was not greatly affected by increasing Mg^{2+}, even to 10 times the zinc concentration. Also, because the PTS assay is carried out with permeabilized cells, any effects of zinc on glycolysis and phosphoenolpyruvate supply are not of concern. The inhibitory effects of zinc were reduced by addition of sulfhydryl agents such as dithiothreitol. However, such reduction does not necessarily indicate that Zn affects targets by associating with sulfhydryl groups. It may only indicate that Zn bound

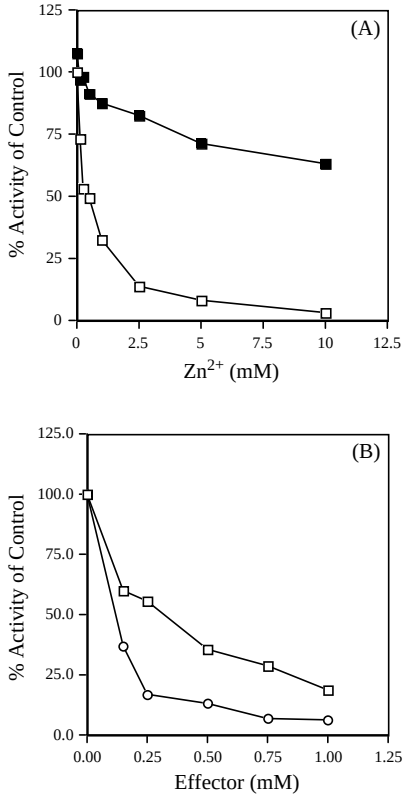

Fig. 4. (A) Inhibition of glucose-PTS activity of permeabilized cells of *S. mutans* GS-5 either washed (■) or not washed (□) after zinc sulfate treatment. 100% activity = 1.44 μmol pyruvate/mg cell dry weight/h. (B) Comparison of effectiveness for inhibition of glucose-PTS of Zn added as the sulfate salt and in zinc-citrate complexes. Symbols in panel B indicate cells treated with Zn sulfate (●) or Zn citrate (○).

to the sulfhydryl agents is not an effective inhibitor.

The results of experiments to determine zinc sensitivity of the sucrose-PTS showed a sensitivity essentially the same as that of the glucose-PTS.

Inhibition of glycolytic enzymes

Zinc is known to inhibit certain glycolytic enzymes. The example shown in Fig. 5 is for inhibition of pyruvate kinase. Scheie et al. (26) previously showed that zinc can also inhibit aldolase and glyceraldehyde-3-P dehydrogenase of *S. sobrinus*. Their results were interpreted in terms inhibition of metalloenzymes with sulfhydryl groups to which zinc can bind. This interpretation is reasonable. However, as shown here, relatively high concentrations of zinc were required for inhibition of pyruvate kinase. The 50% inhibitory concentration was somewhat greater than 1 mM, even under enzyme-limiting assay conditions. Thus,

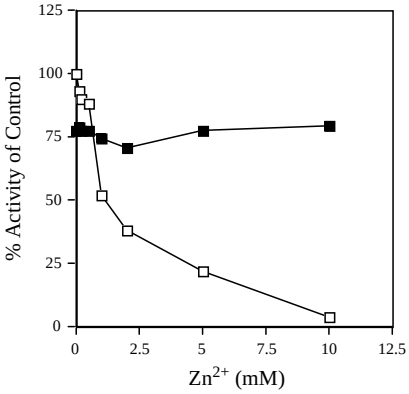

Fig. 5. Inhibition of pyruvate kinase activity of permeabilized cells of *S. mutans* GS-5 either washed (■) or not washed (□) before pyruvate assay. 100% activity = 0.015 μmol/mg cell dry weight/min. The suspension density was 1 mg cell dry weight per ml based on the initial weight of the cells permeabilized.

pyruvate kinase appears to be a secondary target for zinc inhibition of glycolysis, while the PTS system was a primary target because of its membrane location and its greater sensitivity to zinc.

The data presented in Table 1 indicate responses of intact cells to zinc inhibition of glycolysis reflected in changes in levels of glycolytic intermediates. Control data are presented for glycolysis of cells not exposed to zinc and for cells exposed to 0.10, 0.25 or 1.0 mM $ZnSO_4$. Data for starved cells not given glucose are presented to illustrate differences in intermediate levels between the two states of glycolytic activity. Three separate experiments were carried out. In each, cells were given glucose in excess, except for the starved cells, and allowed to reach a state of steady glycolysis as indicated by a constant rate of acid production. $ZnSO_4$ was then added or not added. When a new steady state of glycolysis was achieved, glycolytic intermediates were extracted from the cells with perchloric acid. Values for intermediate levels in control glycolyzing cells undergoing glycolysis were set as 100% for analyzing data from each of the three experiments. Values for levels of intermediates in the zinc-treated or the starved cells were converted to percentages of control values for each particular experiment.

As expected from the findings for zinc effects on the glucose-PTS, treating glycolyzing cells with zinc sulfate resulted in reduction in levels of glucose-6 phosphate (G6P), although the reduction was not to the low level found for starved cells. There was less effect of zinc treatment on levels of fructose-6-phosphate (F6P) or fructose 1,6 bisphosphate (FBP), although FBP levels were somewhat reduced, suggesting some zinc effect on phosphofructokinase. Combined levels of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P) were somewhat reduced, suggesting some small effect on aldolase. The marked reductions in levels of 3-phosphoglycerate (3PGA) and 2-phosphoglycerate (2PGA), especially for cells exposed to 1.0 mM zinc, suggest inhibitory effects on glyceraldehyde-3-phosphate dehydrogenase. Phosphoenolpyruvate levels were reduced only at 1.0 mM zinc, and actually increased at the lower zinc levels. The known tendency for cells of mutans streptococci is to increase pools of phosphoenolpyruvate plus 2PGA when starved, is indicated by the data presented for starved cells. The reduction of levels of pyruvate in the cells suggests inhibition of pyruvate kinase by zinc, mainly at a level of 1.0 mM. The decline in lactate levels reflects the general inhibition of glycolysis because lactate as the main endproduct under conditions of glucose excess. The amounts of lactate shown are high and would include lactate produced during the time taken to achieve steady-state glycolysis. Lactate is a weak acid and can move into and out of the cell to follow the pH gradient across the membrane. In separate experiments, it was determined that lactate dehydrogenase of the organism was not inhibited by zinc at the concentrations used here (data not shown). Overall, the changes in levels of glycolytic intermediates are in line with knowledge of the inhibitory levels of zinc for individual glycolytic enzymes. The main zinc-sensitive enzymes based on results of studies with isolated enzymes or permeabilized cells are the PTS, aldolase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase.

Inhibition of alkali production

Previously, Giertsen et al. (12) found that zinc could inhibit alkali (NH_3) production from urea by dental plaque. As shown by the data in Fig. 6(A), urease activity of intact cells of *S. salivarius* was highly sensitive to inhibition by zinc. Moreover, the data of Fig. 6(B) indicate that urease enzymes in permeabilized cells of the organism were even more sensitive to inhibition by zinc. It is well known that urea can enter cells independently of transport systems (4), although streptococci do have an H^+-gated urea transporter, but genes for urea transport systems are generally transcriptionally

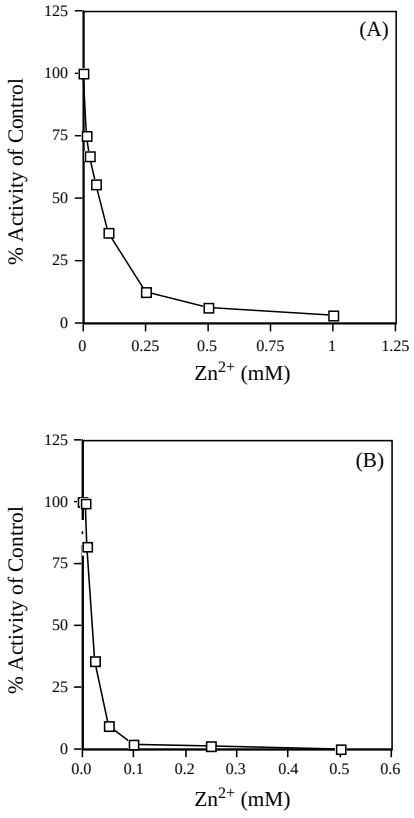

Fig. 6. $ZnSO_4$ inhibition of urease activity of intact (A – 0.4 mg cell dry weight/ml) or permeabilized (B – ca. 0.8 mg cell dry weight/ml) cells of *S. salivarius*. 100% activity = 0.09 μmol ammonia/mg cell dry weight/min for intact cells and 0.37 μmol ammonia/mg cell dry weight/min for permeabilized cells.

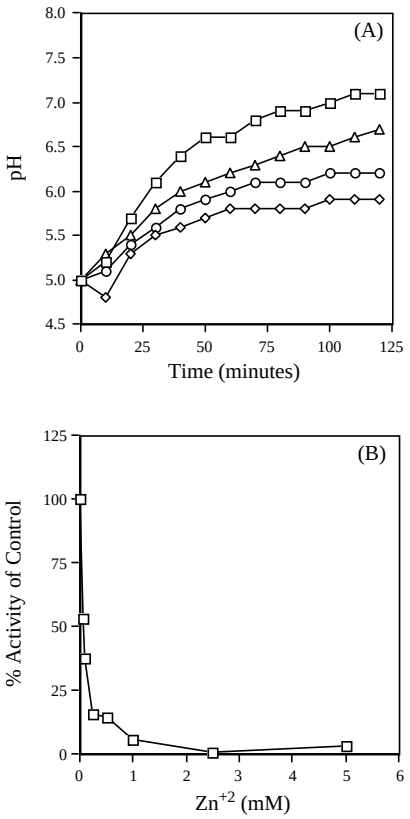

Fig. 7. Zinc inhibition of alkali production (pH rise) from arginine by intact cells of *S. rattus* in suspensions (A – 0.4 mg cell dry weight/ml) and of activity of arginine deiminase enzyme in permeabilized cells (B – ca. 0.8 mg cell dry weight/ml). 100% activity for permeabilized cells = 5.8 μmol citrulline produced/mg cell dry weight/min. Symbols in panel A: -□- control, -△- 0.1 mM Zn^{2+}, -○- 0.5 mM Zn^{2+} and -◇- 1.0 mM Zn^{2+}.

upregulated, mainly during times of nitrogen starvation. It seems that sensitivity of ureolysis by intact cells to zinc can be interpreted mainly in terms of zinc inhibition of urease enzyme.

As shown by the data presented in Fig. 7(A), zinc can inhibit rises in pH due to ammonia production from arginine by intact cells of *S. rattus*. Zinc is also highly inhibitory for the arginine deiminase enzyme of permeabilized cells (Fig. 7B). Attempts to show that zinc inhibited arginine uptake did not indicate major inhibition when cells were initially exposed to zinc. However, zinc inhibition of arginine deiminase enzyme in oral streptococci would result in reduced levels of ornithine, the substrate for arginine-ornithine antiport, which is a major means for the uptake of arginine for catabolism. Arginine deiminase is the signature enzyme of the arginine deiminase system and the first enzyme in the pathway of catabolism. Thus, inhibition of the enzyme would effectively stop substrate flow through the system and production of ornithine for arginine-ornithine antiport.

S. rattus can grow in complex medium with arginine as the sole catabolite. This growth could be inhibited with zinc. However, we encountered previously documented (18) problems in determining minimal inhibitory concentrations for zinc inhibition of growth in complex media because of binding of the metal to medium components. Zinc citrate proved to be a more effective inhibitor. The minimal inhibitory concentration for growth of the organism in TY medium with 1% arginine was about 0.75 mM even for zinc citrate and about 3 times higher for zinc sulfate. These findings indicate that a major advantage of using zinc citrate rather than zinc chloride or sulfate as an antimicrobial is that the citrate prevents sequestering of the metal by sulfhydryl compounds in the environment. Sodium citrate itself had no inhibitory effect on *S. rattus* growth in TY-arginine medium. Assessments of minimal inhibitory concentrations for zinc inhibition of growth of *S. mutans* GS-5 in TYGlucose medium indicated that zinc citrate was more effective than the sulfate salt, with minimal inhibitory concentrations of about 1 and 5 mM, respectively.

Zinc killing

Zn^{2+} alone did not appear to be bactericidal for oral streptococci even at levels of 20 mM as the sulfate or chloride salts, in contrast to Fe^{2+} and Cu^{+}, which are highly toxic for mutans streptococci at these levels, even under anaerobic conditions (11). Zinc citrate at high levels of 10 or 20 mM was lethal even for cells in biofilms. However, the bactericidal effect was minimal. For example, killing of *S. mutans* cells in biofilms by 20 mM zinc citrate was biphasic, with almost no killing during the first hour of exposure and then killing with a D value (time for killing of 90% of the population) of approximately 1 h. The nature of the lethal effect is not clear, but it requires zinc levels higher than those for the other inhibitory actions described here and probably higher than those achievable in biofilms in the human mouth.

Discussion

A number of questions, listed below, regarding zinc action can be answered on the basis of the results presented here.

Does zinc affect acid tolerance as well as acidogenesis?

Zinc does affect acid tolerance, apparently by inhibiting the action of the transmembrane proton-translocating F-ATPase. However, the inhibition is reversible. Zinc cations may compete for metal-binding sites of the enzyme. As shown previously (27), there is a hierarchy of cation specificity for F-ATPase activity, Mg^{2+} being the best for catalysis of ATP hydrolysis and Zn^{2+} much less effective. However, zinc could also inhibit F-ATPases by binding to sulfhydryl groups. Zinc has been found to have potent inhibitory actions for a number of transmembrane ion and proton channels (20). The finding that F-ATPases of oral streptococci are zinc sensitive is therefore not surprising. However, it at least partially explains the reduction by zinc of acid tolerance of glycolysis by intact cells. As mentioned, reductions in the rate of ATP supply may also be important in zinc reduction of acid tolerance.

Is zinc effective against biofilms?

Yes, but as is the case for many antimicrobials, zinc potency is significantly reduced due to high biomass concentrations in the films. Therefore, although the standard inhibitory effects of zinc can be demonstrated for biofilms, the concentrations of the agent required are higher than those for similar effects against cells in suspensions. This biomass effect suggests that zinc binds both to sites effective for inhibition and to ineffective sites.

Can zinc be bactericidal rather than just bacteriostatic?

It seems that Zn^{2+} alone probably can be only bacteriostatic – inhibition of glycolysis, PTS, and F-ATPase were reversible. However, it does seem that zinc can enhance the bactericidal actions of other agents. Its interactions with the bactericidal agent triclosan have been well described in the literature (9). Izaguirre-Fernández et al. (16) found that zinc and fluoride together can kill oral bacteria, provided that the mix is used at low, potentially lethal, pH values. In other words, the action appears to be primarily an enhancement of acid killing. It seems that these lethal actions reflect only zinc enhancement of killing by another agent and not the bactericidal capacity of zinc itself. The major exception is killing associated with treating cells with very high levels of zinc citrate. However, the killing was slow and probably not relevant to damage to oral streptococci *in vivo*. It may be related to known bactericidal effects of mM levels of Fe or Cu cations (10) even under anaerobic conditions in which reactive oxygen species could not be formed.

What are the main mechanisms for zinc inhibition of alkali production?

Zinc was found in our work to be a potent inhibitor for the catabolic enzymes arginine deiminase of *S. rattus* and urease of *S. salivarius*. The inhibition of the arginine deiminase enzyme would be sufficient basis for inhibition of arginolysis and for inhibition of growth with arginine as primary catabolite. Urea is not an effective catabolite for growth, although it can provide nitrogen for biosynthesis (6). It is well known that urea can enter cells without the need for a transport system, although many cells do have transport systems for urea uptake, presumably supplementing the general diffusion of the solute across the cell membrane. However, the inhibition of urease enzyme reported here would be sufficient for inhibition of alkali production from urea. We found similar inhibitions of purified jack bean urease and the enzyme from *Bacillus pasteurii*. The finding that zinc can inhibit urease, even though a transport system for urea is not required for catabolism, suggests that zinc can gain access to the cytoplasm and can affect cytoplasmic enzymes.

The results of previous studies reviewed by Cummins (8) indicates that zinc is readily absorbed by cells of mutans streptococci from the sulfate salt or from chelated forms such as zinc citrate or zinc ethylene diaminetetraacetate. Current indications are that zinc taken up into the cytoplasm of bacterial cells is mainly bound (23), so the distribution of zinc among various binding sites within the cell would be determined mainly by affinity. The data presented here indicate that targets such as urease and arginine deiminase must be able to compete for zinc because their actions in intact cells could be inhibited by the metal. Zinc is taken up by bacterial cells by a variety of transport systems (13), one of which is induced by peroxides (11). Thus, zinc would be expected actually to enter the cytoplasm, and cytoplasmic enzymes, including glycolytic enzymes, appear to be accessible targets. Even for zinc inhibition of membrane enzymes such as the F-ATPase and the phosphotransferase system, access to inhibitory sites is likely to be from the cytoplasmic side. Both enzyme systems catalyze transphosphorylation, which is commonly sensitive to zinc inhibition (20). The general picture of zinc antimicrobial action then is one that involves multiple targets within cells.

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