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Effect of Trace Metals on Growth of *Streptococcus mutans* in a Teflon Chemostat

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Correlations between the presence of certain trace metals in dental enamel or in drinking water and the incidence of human dental caries have been demonstrated; therefore, the effects of several trace metals on growth of the cariogenic organism *Streptococcus mutans* OMZ176 were determined. For continuous growth in a chemically defined medium (treated with Chelex-100 to lower trace metal contamination and supplemented with high-purity trace metal salts) used in a chemostat constructed of Teflon, *S. mutans* required input of carbon dioxide and supplementation with magnesium (126 μM) and manganese (18 to 54 μM). Addition of iron (3.6 μM) increased the level of steady-state growth by a factor of 2.8 (stimulation index [SI]); zinc at 0.4 μM nearly doubled equilibrium growth (SI = 0.9). Higher concentrations of iron and zinc (5.4 and 0.8 μM, respectively) were less stimulatory (SI values of 1.95 and 0.3, respectively). Small (but statistically significant) increases in steady-state growth were effected by cobalt (SI = 0.3 at 5.1 to 20.4 μM) and tin (SI = 0.4 at 5.1 to 10.2 μM). These data suggest nutritional requirements for these metals. Copper at a concentration of 0.16 μM was inhibitory. These results show significant effects of these metals on growth of *S. mutans* and may confirm epidemiological evidence suggesting a role for certain trace metals in the incidence of dental caries.

A relationship between the prevalence of human dental caries and certain trace elements (other than fluoride) has been established. A low incidence of caries has been related to increased concentrations of aluminum, iron, selenium, and strontium in human dental enamel; a high incidence of caries has been associated with elevated levels of manganese, copper, and cadmium (7, 14). A decreased incidence of caries has also been noted with increased amounts of calcium, magnesium, strontium, and lithium in dental plaque (17, 18). Acid formation by dental plaque has been decreased by rinsing the mouth with solutions of zinc, aluminum, and iron; however, magnesium has been shown to increase acid formation (15).

A correlation between incidence of caries and trace elements in food and drinking water has been considered. For example, calcium, magnesium, molybdenum, and vanadium concentrations were found to be high in water samples from areas characterized by a low incidence of caries (12), whereas copper and manganese have been found to be associated with development of caries (1). Reduced incidence of caries in humans also has been related to strontium in drinking water (8). Studies of caries in experimental animals have yielded conflicting reports (possibly due to variations in protocols); nevertheless, in rats, treatment with strontium (posteruptively, in drinking water) (2) and oral administration or topical application of zinc lower the incidence of caries (3). The epidemiological and clinical evidence presented in these references corroborate that certain elements may enhance susceptibility to caries, whereas other elements may have cariostatic properties.

The mechanism by which trace elements exert an effect on the pathogenesis of caries is unknown; however, several potential functions in cariogenesis may be suggested: adherence of bacteria to the tooth surface, production of host resistance factors, alteration of tooth structure, and effects on microbial growth and metabolism. The etiological agent(s) of caries has not been established; nevertheless, evidence indicates that *Streptococcus mutans* plays an important role in the development of caries (13). This organism's requirements for and sensitivities to trace elements may have significance in the etiology of dental caries.

Laboratory studies on the effects of trace elements on *S. mutans* growth have been undertaken to a limited extent. In medium containing the chelating agent EDTA (added in an effort to render certain metals unavailable to the organism), several strains of *S. mutans* require manganese and possibly calcium and magnesium (4).

In another study, relatively high concentrations of 12 elements were added to *S. mutans* growing in a complex medium; all additions were shown to inhibit growth (11). Other experiments showed that molybdenum increases cell yields (6), and zinc inhibits plaque formation on nichrome wires by *S. mutans* (3). Growth of *Streptococcus salivarius* is stimulated by the addition of calcium and magnesium, whereas zinc and manganese are inhibitory; however, concentrations below 0.1 mM were not previously tested (5).

In the present work, the growth response of *S. mutans* OMZ176 to seven metals is reported. A chemostat constructed of Teflon was used for continuous cultivation in a chemically defined medium that was treated before use to lower contamination with trace metals and supplemented with high-purity metal salts. Teflon was utilized to control leaching of trace elements from chemostat components. Addition of certain metals was required for growth, and concentration-dependent stimulatory or inhibitory responses to other metals (at concentrations of a few micromolar or less) were noted. The presence and concentration of these micronutrients may influence the development of caries.

(A preliminary report of these findings has already appeared [H. Aranha, R. C. Strachan, J. E. L. Arceneaux, and B. R. Byers, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, D-64, p. 53].)

MATERIALS AND METHODS

Bacterial strain and culture medium. *S. mutans* OMZ176 (Bratthall serotype d) was obtained from G. D. Shockman, Temple University Health Science Center, Philadelphia. The organism was kept in a lyophilized state; working stock cultures were maintained in Todd-Hewitt broth.

The chemically defined medium (FMC) described by Terleckyj et al. (21) was used with the following modifications. The glucose concentration was decreased to 0.8%. Sodium carbonate was eliminated from the medium and replaced by a continuous input of filter-sterilized CO_2 at a flow rate of 50 ml/min. To lower contamination of the medium with trace metals, we treated a 10× solution of all ingredients (except the amino acids, vitamins, and nucleic acid bases) by previously described methods (9) with Chelex-100 (Bio-Rad Laboratories). For use, the solution was reconstituted with high-purity water (obtained from a Millipore RO-40/Milli-Q system); the amino acids, vitamins, and nucleic acid bases were then added, the pH was adjusted to 6.5, and the medium filter sterilized. Unless otherwise stated, the sterile medium was supplemented with high-purity (Johnson-Matthey) $MgSO_4 \cdot 7H_2O$ (420 μM magnesium), $MnSO_4 \cdot 5H_2O$ (180 μM manganese), and $FeSO_4 \cdot 7H_2O$ (18 μM iron). As desired, high-purity salts of other trace metals ($ZnSO_4 \cdot 5H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $SnCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$) were also added.

Continuous cultivation in the Teflon chemostat. The chemostat constructed of Teflon for trace metal studies of *S. mutans* has been described previously (19). Inocula for the chemostat were prepared as follows. An 18-h culture of *S. mutans* grown anaerobically (Forma Scientific Anaerobic System model 1024) at 35°C in the modified FMC medium (adjusted to pH 7.5) was harvested by centrifugation, washed twice with modified FMC medium, and used to inoculate a 600-ml volume (10^7 cells per ml) in the chemostat culture chamber. The pH in the chemostat was maintained at 6.5 by automatic addition of 10 N KOH, and the incubation temperature in the chemostat was 35°C. Input gas was CO_2 (50 ml/min); this effected a dissolved oxygen concentration which was less than 5% that in medium saturated with air. When growth reached the logarithmic phase, the medium input system was activated. Periodic sampling was done by withdrawing a sample from the culture chamber. Growth was determined by dry-weight measurements (19).

Determination of the effect of metal ion addition: calculation of the SI. After steady-state growth was attained in the absence of the test metal, the effect of the metal was then determined by simultaneous addition of the metal (as a filter-sterilized solution of the high-purity metal salt) to the medium reservoir and to the culture chamber. Routinely, the effects of various concentrations were determined by stepwise increases; equilibrium growth was reestablished after each increase in metal concentration. The stimulation index (SI) for each concentration of the metal was calculated by the formula: $SI_x = (DW_x/DW_0) - 1$, in which SI_x = SI at x concentration of the metal, DW_0 = steady-state dry weight in the absence of the metal, and DW_x = steady-state dry weight attained at x concentration of the metal. An SI value of 1 represents a doubling of the steady-state dry weight.

RESULTS

Growth of *S. mutans* OMZ176 in the Teflon chemostat: requirements for CO_2, magnesium, and manganese, and effect of dilution rate. Analyses (by atomic absorption spectroscopy) of the ingredients of the chemically defined FMC medium for the presence of the metals under study showed various levels of contamination; sodium carbonate contained the highest levels (data not shown). For cultivation in the Teflon chemostat, the sodium carbonate was eliminated from the medium, and the remaining ingredients (except the vitamins, amino acids, and nucleic acid bases) were treated with Chelex-100. These procedures lowered the concentrations of magnesium, manganese, iron, cobalt, zinc, tin, and copper below the limits at which these elements were detectable by atomic absorption spectroscopy (0.3, 0.4, 0.7, 0.9, 0.1, 10, and 0.5 μM, respectively). The carbonate source was replaced with a continuous flow of CO_2; little or no growth occurred when the input gas was N_2 (dissolved oxygen level < 1%), suggesting that carbonate was required for growth in the modi-

fied FMC medium. Tests of the effects of oxygen on growth in the chemostat (with various mixtures of air-CO_2) showed that growth was inhibited at dissolved oxygen concentrations above 6% that in medium saturated with air. For subsequent work, input of CO_2 (50 ml/min) was used to satisfy the carbonate requirement and to maintain a dissolved oxygen level of less than 5%.

In the modified FMC medium, no growth occurred without a magnesium supplement. Anaerobically incubated batch cultures showed that 126 μM magnesium was the minimal concentration required for maximal growth. For subsequent continuous cultivation experiments, the medium contained 420 μM magnesium. As was reported for *S. mutans* BHT (19), strain OMZ176 also required manganese for growth. To satisfy this requirement, we modified the medium so that it contained 180 μM manganese, although 18 μM manganese allowed continuous growth at a low population density and maximum growth responses occurred at 54 μM manganese (R. C. Strachan, unpublished data).

Optimal growth was achieved at relatively low dilution rates; a dilution rate above 0.15/h decreased the dry weight by 28% (Table 1). For all subsequent work, a dilution rate of 0.13/h was used (steady-state mean generation time, 5.3 h).

Stimulation of growth by iron, zinc, cobalt, and tin. *S. mutans* OMZ176 was capable of continuous growth in the modified FMC medium supplemented only with magnesium and manganese; however, addition of certain other metals increased the dry weight of steady-state cultures. The effect of various concentrations of a test metal was determined by stepwise increases in metal concentration (steady-state growth was reestablished after each increase). An SI for each concentration was calculated by comparison of the steady-state dry weight achieved in the absence of the metal with the steady-state dry weight observed after addition of the metal (see Materials and Methods).

Supplementation with iron produced marked stimulation of the level of equilibrium growth (Fig. 1). Iron at 1.8 μM more than doubled the steady-state growth, and 3.6 μM iron caused an increase of nearly threefold in the growth level (SI values of 1.45 and 2.8, respectively); however, 5.4 μM iron was less stimulatory (SI = 1.95). These data suggest a nutritional requirement for iron in *S. mutans* OMZ176; the optimal iron concentration was near 3.6 μM. A requirement for zinc was also indicated by results (Fig. 1) showing that 0.4 μM zinc nearly doubled the level of steady-state growth, whereas 0.8 μM zinc produced less stimulation (SI values of 0.9 and 0.3, respectively).

TABLE 1. Effect of dilution rate on growth of *S. mutans* OMZ176 in modified FMC medium in a Teflon chemostat[a]

Dilution rate/h	Dry wt (mg/ml)
0.06	0.05
0.10	0.72
0.15	0.75
0.18	0.54

[a] The medium was treated with Chelex-100 and contained 420 μM magnesium, 180 μM manganese, and 18 μM iron. The CO_2 input was 50 ml/min, and the dissolved oxygen was less than 5%. The pH was maintained at 6.5, and the incubation was at 35°C.

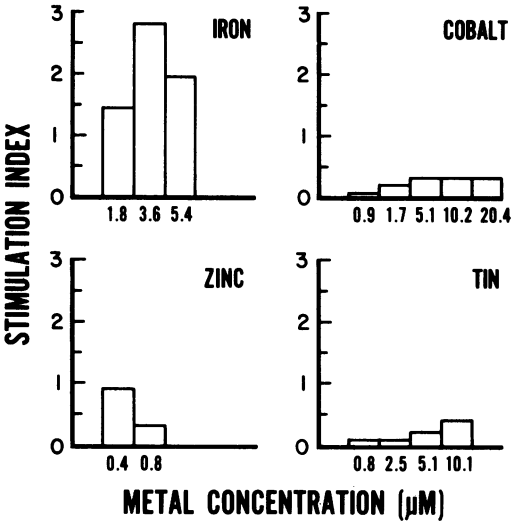

FIG. 1. Stimulation of steady-state growth of *S. mutans* OMZ176 by addition of various metals during continuous cultivation. An SI of 1 represents a doubling of steady-state growth.

Whereas a small increase in the level of steady-state growth was caused by addition of cobalt (SI = 0.3 at 5.1 to 20.4 μM cobalt), a low cobalt concentration (0.9 μM) produced little increase in dry weight (Fig. 1). These data suggest an increase in the population density at cobalt concentrations of 5.1 μM or above. A similar pattern was noted for tin (Fig. 1). A concentration of 10.1 μM tin produced an SI of 0.4; concentrations below 5.1 μM caused little change in the dry weight. The stimulation evidenced by additions of cobalt and tin was demonstrated by Dunnett's procedure (10) for comparison of means (α = 0.05) to be statistically significant.

Inhibition by copper. Addition of copper (0.16 μM) to steady-state cultures caused a rapid decrease in the population density (Fig. 2). Removal of copper from the incoming medium (with resulting dilution of copper in the culture

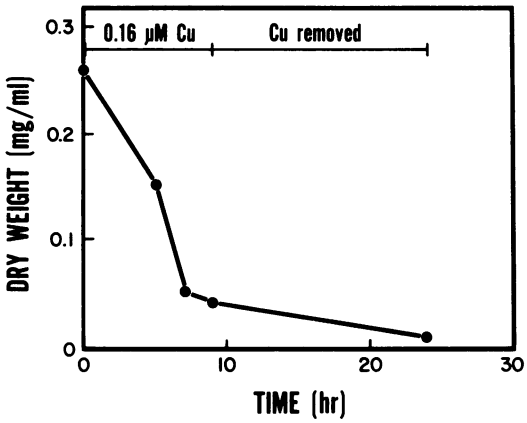

FIG. 2. Effect of copper on *S. mutans* OMZ176. Copper (0.16 μM) was added to the steady-state culture (and to the incoming medium) at time zero. Copper was removed from the incoming medium at 9 h.

chamber) did not allow the culture to return to its initial growth level during 14 h.

DISCUSSION

For growth in a chemically defined (Chelex-100–treated) medium in a Teflon chemostat, *S. mutans* OMZ176 required carbonate (CO_2 input), magnesium, and manganese. Definite stimulation of growth was noted upon addition of low concentrations of iron and zinc; small (but statistically significant) stimulation of growth was effected by additions of cobalt and tin, suggesting nutritional requirements for these elements. Although magnesium, manganese, iron, zinc, and cobalt are known to have biological activity, a possible nutritional requirement for tin was not predicted. Tin was tested because it is a component of some dentifrice preparations. Growth of the organism without added iron, zinc, cobalt, or tin may have been supported by residual amounts of these metals in the medium.

The response of *S. mutans* to iron and zinc may be typical of this organism's response to a number of trace metals above-optimum concentrations of which may be inhibitory. No effort was made to establish toxic concentrations of the metals; however, the highest tested concentrations of both iron and zinc were less stimulatory than were lower levels. Inhibition of growth by high concentrations of certain metals is likely; for example, zinc is inhibitory (11) at a concentration 15 times greater than the level tested in the present studies. Critical to an in vivo effect of a metal ion is the concentration of the metal in the microenvironment of the organism; extracellular macromolecules may control the concentrations and types of ions that reach the cell. Plaque has been shown to selectively bind certain ions (17, 18), and this property may partially regulate the metal ion environment of the organism within the oral cavity.

Integration of data presented here with published information on the cariogenic or cariostatic effects of the metals is difficult, but some interesting speculations are possible. Both manganese and copper (when present in tooth enamel or in drinking water) have been related to an increased incidence of caries (1, 7, 14). *S. mutans* OMZ176 required manganese at a fairly high concentration in our experiments (which might suggest a cariogenic potential for this metal); however, copper was inhibitory at a low concentration. These data suggest that the cariogenic property ascribed to copper may be due to an effect on development of caries at a point other than the growth and metabolism of *S. mutans*. Toxic levels of copper may not reach the organism.

The apparent carbonate and magnesium requirements of *S. mutans* may be important in determining the initial site of carious attack. Pockets of high magnesium and carbonate occur in dental enamel, and it has been suggested that these areas may be highly susceptible to carious lesions (16). Carbonate reduces the number of amino acids needed by *S. mutans* (20). Therefore, areas of the teeth rich in these two nutrients may favor growth of *S. mutans*. An additional consideration in the pathogenesis of caries is the presence of trace elements in dental plaque. Increased magnesium in plaque may be associated with reduced incidence of caries; however, calcium, strontium, and lithium in plaque have also been implicated in cariostasis (17, 18). Possible cariostatic properties of these elements may override magnesium stimulation because rinsing the mouth with magnesium has been shown to increase acid production by dental plaque (15).

The cariostatic potential of zinc has been demonstrated by recent studies in which 62 μM zinc (the lowest concentration tested) was shown to inhibit growth of *S. mutans* in batch cultures, and addition of zinc to drinking water was shown to reduce the incidence of caries in rats (3). In the present studies, 0.8 μM zinc was less stimulatory than 0.4 μM zinc; therefore, the cariostatic property of zinc may be due to toxicity of the metal at concentrations in excess of the required level.

The results presented here show significant effects of magnesium, manganese, iron, zinc, cobalt, tin, and copper on *S. mutans* growth and may confirm epidemiological evidence indicating a role for certain trace metals in the incidence of dental caries. The continuous culture system used here should facilitate demonstra-

tion of unsuspected nutritional requirements for (or effects of) other trace elements in *S. mutans*. Additional elements with known cariostatic or cariogenic properties are under study.

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