

Effect of fluoride on glucose incorporation and metabolism in biofilm cells of *Streptococcus mutans*

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The aim of this study was two-fold: firstly, to study the effect of high fluoride concentrations on carbohydrate metabolism in *Streptococcus mutans* present in biofilms on hydroxyapatite; and, secondly, to evaluate the effect of fluoride-bound hydroxyapatite on lactic acid formation in growing biofilms of *Strep. mutans*. Biofilms of a clinical strain of *Strep. mutans* on saliva-coated hydroxyapatite beads were incubated with sodium fluoride over a wide range of concentrations. At high fluoride concentrations (>10 mM) the incorporation of [¹⁴C]-labeled glucose decreased by 80–85%, at both pH 7.0 and 5.6. At lower fluoride concentrations, the effect of fluoride on the incorporation of labeled glucose was pH-dependent in both biofilm cells and in planktonic cells. At pH 7.0, fluoride at concentrations <10 mM had little or no effect. Pretreatment of hydroxyapatite discs with fluoride varnish (Fluor Protector) or fluoride solutions caused a statistically significant reduction of lactic acid formation in associated, growing biofilms of *Strep. mutans*. Fluoride varnish and 0.2% (47.6 mM) sodium fluoride solution exhibited a statistically significant inhibitory effect on lactate production.

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The formation on tooth surfaces of bacterial biofilms, called dental plaque, is a prerequisite for the development of both dental caries and periodontal disease. Bacterial carbohydrate metabolism in these biofilms, with concomitant release of organic acids, is the cause of dental caries. Among bacterial species in dental plaque, mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) are considered the main etiologic agents of enamel caries.

Fluoride plays an important role in dental caries prevention, mainly due to its effect on the calcified tissues of teeth. However, an additional important preventive effect of fluoride is its ability to reduce acid formation in some bacterial species of dental plaque e.g. mutans streptococci. In sensitive bacteria, fluoride can impair glycolysis by inhibition of enolase activity. Enolase, one of the enzymes of the Embden-Meyerhof pathway, converts 2-phosphoglycerate (2PGA) to phosphoenolpyruvate (PEP) (1). Inhibition of enolase, leading to a reduced cellular level of PEP, also inhibits sugar uptake via PEP-dependent phosphotransferase system. A further effect of fluoride on bacterial metabolism is a direct inhibitory effect on the membrane-associated proton-pumping H⁺-ATPase (2). KANAPKA & HAMILTON (3) and HAMILTON (4) have shown a reduction in the cellular level of PEP and ATP and an increased content of 2PGA in *Streptococcus salivarius* after addition of sodium fluoride. It has been shown that for *Strep. mutans* under glucose excess and at low environmental pH, the accumulation of

biofilm cells on fluoride-bound hydroxyapatite was significantly reduced (5).

To date, however, surprisingly few reports have described the effect of high fluoride concentrations (above 5 mM) on carbohydrate metabolism in oral bacteria, even though high concentrations are used clinically. It has been stated (6) that the optimal concentration of sodium fluoride for topical application is not known, but most of the available clinical evidence supports the use of 2% (47.6 mM) aqueous solution of fluoride and that the preferred stannous fluoride solution is 1905 mM. WOOLLEY & RICKLES (7) showed that a daily application of 2% sodium fluoride solution reduced the pH drop in plaque by 0.5-pH unit. After 1 to 3 wks of daily 5-min applications of 1% sodium fluoride gel (238 mM), a decrease of approximately 60% in sucrose-induced lactate production in plaque was reported by BROWN *et al.* (8).

A large number of reports has shown that the sensitivity of bacterial cells to fluoride is markedly affected by the environmental pH. At low environmental pH, a pH gradient (Δ pH) will be established between the intracellular pH (pH_i) near neutrality and the extracellular pH (pH_e). The pH gradient will increase as the pH_e decreases. The pH gradient will cause fluoride to accumulate intracellularly against a concentration gradient (5, 9–11). As a consequence, the effect of fluoride on carbohydrate metabolism at external pH near neutrality is low or non-existent. The energy-independent nature

of fluoride uptake has been demonstrated in *Streptococcus sanguinis* (formerly *Strep. sanguis*) by KASHKET & RODRIGUEZ (12) and in *Strep. mutans* by WHITFORD *et al.* (13).

Despite the extensive literature on the effects of fluoride on carbohydrate metabolism in oral bacteria, there are few reports on the effect of fluoride on bacteria present in biofilms. Furthermore, little is known of the possible effects of fluoride released from a surface on carbohydrate metabolism in associated biofilms of oral bacteria.

This work was initiated to study the sensitivity of *Strep. mutans* to fluoride at concentrations up to or approaching those used for topical application, with special reference to the effect on fluoride sensitivity of fluoride concentration, external pH and metabolic state. Our own preliminary studies had disclosed that high fluoride concentrations counteracted the well-documented pH-effect on bacterial sensitivity to fluoride.

A further aim was to study the effect of fluoride-bound hydroxyapatite on associated biofilms of *Strep. mutans*.

Material and methods

Bacteria and growth conditions

A clinical isolate of *Strep. mutans*, strain JB 19, originally isolated from an exposed root surface, was used. Bacteria stored at -70°C were used as inoculum for cultures grown anaerobically at pH 6.8 in glucose-proteose-peptone medium in a stirred 1-l fermenter with pH control as described earlier (14). Exponential phase cells were harvested, washed and stored at -70°C until used. It is noteworthy that the strain used had been subcultured only twice since first isolated.

Preparation of biofilm on hydroxyapatite beads

Biofilms were prepared on hydroxyapatite beads, Macro-Prep type II, 80 μm in diameter, (HA beads). Hydroxyapatite saliva coating was prepared by incubation of 20-mg portions of HA beads in 1 ml clarified saliva by inversion for 1 h at room temperature. The beads were then washed twice in cold 1 mM K_2HPO_4 , 1 mM MgCl_2 , 50 mM KCl and 1 mM CaCl_2 , pH 6.5 (buffered KCl). Saliva-coated HA beads were, without delay, incubated with *Strep. mutans* JB 19, in 6 ml buffered KCl for 1 h at room temperature with continuous shaking. The mean concentration was 1.5×10^8 cells per ml. After sedimentation of the beads, samples were removed from the supernatant and from the original cell suspension, and used for viable counts on blood agar plates after serial dilutions of the samples. The plates were incubated anaerobically for 48 h. The amount of bacteria adhering to the HA beads was determined from the difference in the concentration of cells before and after incubation with saliva-coated HA beads. The beads with adhering bacteria were washed twice in buffered KCl prior to experiments.

Saliva preparation

Whole paraffin-stimulated saliva, collected in beakers on ice from four healthy individuals, was clarified by centrifugation

(10,000 $\times g$, 30 min, 4°C). The supernatant samples were pooled, aliquoted and frozen at -20°C before the assay.

Preparation of hydroxyapatite discs

Hydroxyapatite discs were used in order to study the effect of treatment of hydroxyapatite with fluoride varnish on glucose metabolism in associated biofilms of *Strep. mutans*. The HA discs were manufactured by pressing hydroxyapatite powder, HTP gel, in a steel cylinder at 10 tons per square inch. The discs were then incubated for 3 h at 200°C , washed in sterile water for 30 min, and again incubated for 3 h at 200°C . The total surface area of the discs was 9.8 cm^2 and the mean weight was 0.71 g. The discs were moistened with water, and two layers of varnish were brushed onto each side: a total application of 50 μl of fluoride varnish (Fluor Protector). Discs treated with a placebo varnish, without fluoride, were run in parallel. The varnish was peeled off after 3 h and the discs were incubated in buffered KCl overnight. For comparison, discs were treated with sodium fluoride solutions, 0–47.6 mM, (0–0.2%) for 10 min at room temperature and then washed three times with buffered KCl. Discs were tested in duplicate.

Preparation of biofilm on hydroxyapatite discs

HA discs pretreated with fluoride varnish, placebo varnish or fluoride solutions were incubated with clarified saliva, shaken for 1 h at room temperature, and washed three times with buffered KCl. Without delay, the discs were incubated with a 2-ml suspension of *Strep. mutans* JB 19 (3.3×10^9 cells per ml mean value) in buffered KCl for 1 h at room temperature with continuous shaking and washed three times in large volumes of phosphate-buffered saline (PBS), pH 7.2.

Measurement of glucose incorporation in biofilm cells

The beads with the biofilms were incubated in glass vials for 15 min at 37°C with continuous shaking in 0.02 M potassium-phosphate buffers, pH 5.6 or 7.0 (PP-buffers), containing various concentrations of fluoride (0–200 mM). After incubation D-[U- ^{14}C]glucose was added to give a final concentration of 0.1 mM (0.2 μCi per ml), and incubation was continued for 15 min at 37°C with continuous shaking. The beads were then sedimented and washed twice with ice-cold buffered KCl, and then dissolved in 0.5 ml 2 M HCl. The suspensions were transferred to scintillation vials containing 5 ml scintillation fluid. Radioactivity was measured using a liquid scintillation counter. In one series, the HA beads with the biofilms were washed twice with PP-buffer after incubation with fluoride and then incubated in PP-buffer containing the isotope for 15 min at 37°C .

The period of incubation with labeled glucose was 15 min; ROBERTS *et al.* (15) reported maximum label inside *Streptococcus mitis* after 15 min. Prolonged incubation with glucose actually decreased the amount of label inside the cells.

Measurement of glucose uptake in planktonic cells

The effect of sodium fluoride on the incorporation of glucose in planktonic cells of *Strep. mutans* JB 19 was studied by incubation of *Strep. mutans* suspensions (3.1×10^7 cells per ml, mean value) in PP-buffers containing 0–10 mM sodium fluoride with continuous shaking at 37°C . After 15 min [^{14}C]-glucose was added to a final concentration of 0.1 mM

(0.2 μCi per ml). Glucose incorporation was determined after 15 min incubation at 37°C.

Sugar incorporation was measured using a standard membrane filter technique (15). The samples were removed and rapidly filtered under vacuum through 0.45- μm membrane filters. The filters were immediately washed with 5 ml ice-cold PP-buffers (pH 5.6 or 7.0) and dissolved in 5 ml scintillation fluid by shaking and then the radioactivity was counted.

Measurement of lactic acid production in biofilm cells

Biofilm-coated, fluoride-pretreated HA discs (described above) were incubated for 180 min at 37°C with continuous shaking in a chemically defined growth medium (FMC) (16), containing 4 mg per ml proteose peptone (PP, Difco), and 1% glucose. The pH of the medium was 7.0. Lactic acid in the growth medium was measured with gas-liquid-chromatography (Varian 3400).

Chemicals

The varnish, Fluor Protector, contained polyurethane-based difluorosilane with a fluoride content of 0.1%. The placebo varnish contained polyurethanebase only. Both Fluor Protector and the placebo varnish were supplied by Vivadent-Vivacare, Schaan, Lichtenstein. D-[U- ^{14}C]glucose was obtained from the Radiochemical Centre, Amersham, UK and Insta-GelPlus scintillation fluid from Packard Instrument, Groningen, the Netherlands. The hydroxyapatite beads, Macro-Prep Ceramic Hydroxyapatite, type II and the hydroxyapatite gel, Hydroxyapatite Bio-Gel were from Bio-Rad Laboratories, Hercules, CA, USA. Microporous filters (0.45 μm) were from Millipore, Bedford, MA, USA.

Results

The effect of fluoride on [^{14}C]glucose incorporation in biofilms

Glucose incorporation by biofilms of *Strep. mutans* after preincubation of the biofilm with sodium fluoride (0–200 mM) at pH 5.6 or 7.0 is shown in Fig. 1. Pre-treatment both at pH 5.6 and 7.0 caused a fluoride concentration-dependent reduction in the rate of glucose incorporation. The reduction was most pronounced at pH 5.6, reaching about 85% at 200 mM sodium fluoride. At pH 7.0, the maximum reduction was about 30% at 200 mM sodium fluoride.

Fig. 2 shows glucose incorporation by biofilms of *Strep. mutans* in the presence of external sodium fluoride (0–200 mM). At pH 5.6, maximum inhibition was obtained at concentrations in the range 0–10 mM. At pH 7.0, the reduction in glucose uptake begun to plateau in the range 10–50 mM sodium fluoride. At both pH values, maximum inhibition was 75–80%. At pH 7.0 the rate of glucose uptake in the presence of 10 mM sodium fluoride was reduced by about 15%, while at pH 5.6 the same fluoride concentration caused 75% inhibition.

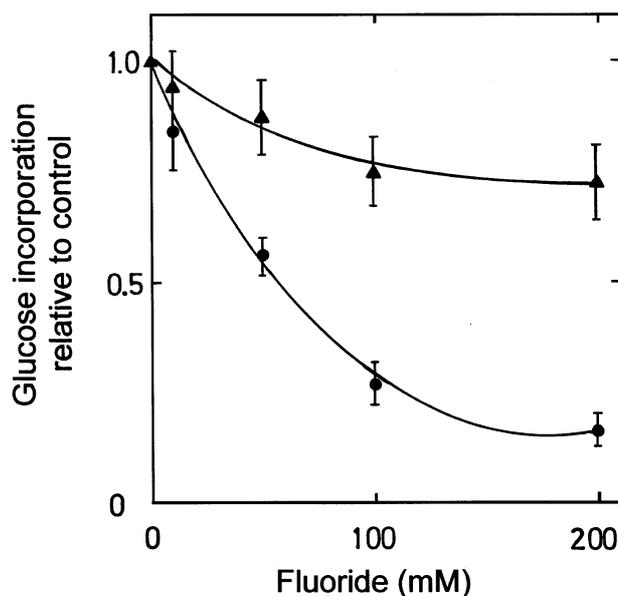


Fig. 1. Incorporation of [^{14}C]glucose by biofilms of *Strep. mutans* JB 19 after preincubation of biofilms with sodium fluoride (0–200 mM) in 0.02 M potassium phosphate pH 5.6 (●) and pH 7.0 (▲). Incorporation was determined at the same pH-values. Mean values and 95% confidence interval are presented. Duplicate determinations in two independent experiments.

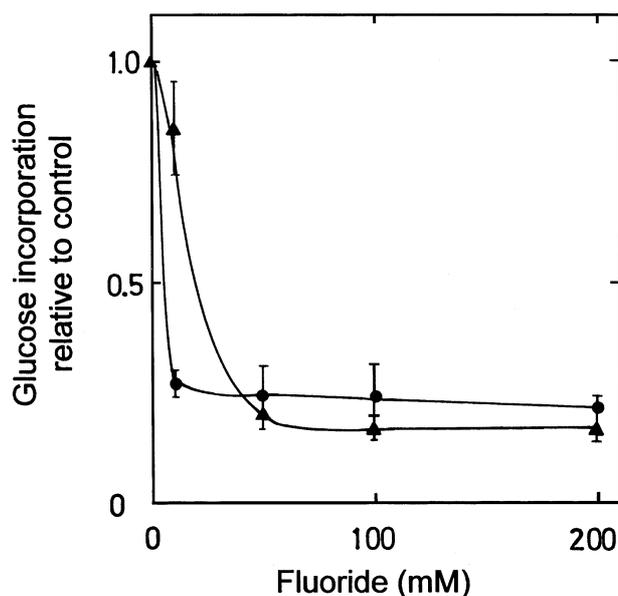


Fig. 2. Incorporation of [^{14}C]glucose by biofilms of *Strep. mutans* JB 19 in the presence of sodium fluoride (0–200 mM) in 0.02 M potassium phosphate at pH 5.6 (●) and pH 7.0 (▲). Mean values and 95% confidence interval are presented. Duplicate determinations in two independent experiments.

The effect of fluoride on [^{14}C]glucose incorporation in planktonic cells

In order to be able to compare our clinical isolate with respect to sensitivity to fluoride with strains of streptococci used by others, experiments were conducted with planktonic cells at low fluoride concentrations. With

respect to fluoride sensitivity, the results presented in Fig. 3 clearly show the resemblance between *Strep. mutans* JB 19 and previously reported strains. At pH 5.6 the rate of glucose incorporation decreased with increasing concentrations of fluoride. At 10 mM sodium fluoride the reduction was near 90%. At pH 7.0 the effect of sodium fluoride in the range 0–10 mM was minimal (Fig. 3).

The effect of fluoride on lactate production in biofilms

Biofilms of *Strep. mutans* prepared on pretreated hydroxyapatite discs were incubated with growth medium, pH 7.0, containing glucose (1%) for 180 min. Pretreatment of the discs with 11.9 or 47.6 mM (0.05% or 0.2%) sodium fluoride or with fluoride varnish (Fluor Protector) reduced lactate production compared to untreated controls (Table 1). Biofilms on discs pretreated

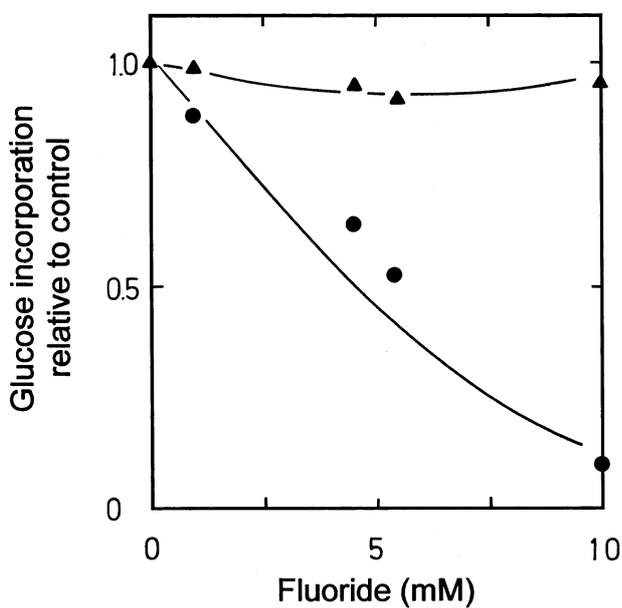


Fig. 3. Incorporation of [14 C]-glucose by planktonic cells of *Strep. mutans* JB 19 in the presence of sodium fluoride (0–10 mM) in 0.02 M potassium phosphate pH 5.6 (●), pH 7.0 (▲). Mean values of three independent experiments.

Table 1

Effect of pretreatment of hydroxyapatite discs with fluoride solutions or fluoride varnish on lactic acid production in biofilms. Biofilms of *Strep. mutans* JB 19 were formed on pretreated discs and incubated in growth medium containing 1% glucose for 180 min. (One-way ANOVA)

NaF	Lactic acid in growth medium mM		P
	Mean ^a	min–max	
0%	2.8	2.5–3.0	0.004
FP-varnish ^b	1.8	1.5–2.0	
0.05%	2.3	2.3–2.4	
0.2%	1.7	1.5–2.0	

^aMean of three experiments.

^bFluor Protector varnish containing 0.1% NaF.

with placebo varnish had essentially the same rate of lactate production as untreated controls. The external pH remained constant during the experimental period. The difference between the fluoride concentrations was statistically significant ($P=0.004$, one-way ANOVA), (Table 1). Pretreatment with fluoride varnish or 0.2% sodium fluoride had a statistically significant inhibitory effect on lactate production, ($P < 0.01$, *post-hoc* test Tukey HSD for unequal N).

Discussion

At low fluoride concentrations (up to 10 mM) our results confirmed earlier observations regarding the effect of fluoride on carbohydrate metabolism in both planktonic and biofilm cells, including the well-documented pH effect. In the presence of high sodium fluoride concentrations, the effect of pH on the sensitivity to fluoride in glycolyzing biofilms of *Strep. mutans* was abolished, while in biofilms exposed to high fluoride concentrations in the absence of an energy source pH strongly affected the sensitivity to fluoride. Our results indicate that at high external concentrations of fluoride the uptake of fluoride in glycolyzing biofilms of *Strep. mutans* is not driven by a pH gradient, while in biofilms exposed to high concentrations of fluoride in the absence of an energy source, fluoride uptake is at least partially dependent on the pH gradient. An incubation period of 15 min was considered appropriate for uptake of fluoride in resting cells on the basis of a report of fluoride uptake in non-metabolizing *Strep. salivarius* (11).

In our study the differences in glucose uptake between biofilm cells and planktonic cells with respect to sensitivity to low concentrations of fluoride were small. By contrast, experiments with planktonic and biofilm cultures of *Strep. sanguinis* grown with sucrose, showed that susceptibility to amine fluoride was less in biofilms than in planktonic cells (17).

Whether or not the observed sensitivity to fluoride at pH 7.0 in glycolyzing biofilms was due to the presence of potassium ions both in washing buffers and during glucose uptake experiments is unclear. MARSH *et al.* (18) claimed that the uptake of fluoride in glycolyzing cells is associated with K^+ movement across the membrane. These authors found that sodium fluoride at a concentration of 24 mM reduced acid production in planktonic cells of *Strep. sanguinis*, washed in KCl, by 80% at pH 7.0 and 8.0. Cells washed in NaCl were immune to the same concentration of fluoride. By contrast, HAMILTON (19) claimed that addition of potassium to cells will reduce the inhibitory effect of fluoride and increase the acidurancy of cells under acidic conditions leading to enhanced acid formation. In addition, GUHA-CHOWDHURY *et al.* (20) reported that fluoride in the presence of sodium rather than potassium ions had a stronger inhibitory effect on net proton movement out of the streptococcal cell. It is noteworthy that potassium is the principle cation in dental plaque fluid (21).

Several studies have shown that fluoride from enamel after topical treatment or from artificial fluorapatite can

influence the metabolism of plaque bacteria at low pH conditions (22–24). Fluoride incorporated in dental enamel may be repository for potentially antimetabolic reserves of fluoride which at low pH levels can be released in concentrations adequate for inhibition of acid production (22). It has been shown that fluoride varnish on the enamel builds up a thick calcium fluoride layer with a high reservoir effect (25, 26). At low environmental pH and under glucose excess, the accumulation of biofilm cells of *Strep. mutans* on fluoride-bound hydroxyapatite rods was significantly reduced ($P < 0.05$ – 0.001) (5). The results of this study demonstrated that fluoride from the substratum may affect fluoride sensitive biofilm cells, but only under conditions of glucose excess and low pH. Our results show that pretreatment of HA discs with fluoride or fluoride varnish could significantly reduce lactate production at pH 7 in growing biofilms of *Strep. mutans*.

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