Inhibition of Acid Production from Oral Bacteria by Fluorapatite-derived Fluoride

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The inhibitory effect of fluorapatite (FAP)-derived fluoride upon resting cell suspensions of Streptococcus mutans *incubated at pH 4.5 and 6.5 was studied using lactic acid production from 0.1% sucrose as an indicator of fermentation activity. Cells incubated with FAP produced significantly less lactic acid than did cells incubated with hydroxyapatite (HAP). Addition of HAP to cell suspensions containing FAP reduced this inhibition, suggesting that dissolution of the FAP was necessary for inhibition. Incubation with low concentrations of NaF showed significant inhibition in cell suspensions incubated with as little as 0.45 μg/mL F at pH 5.0. These results provide further support to the hypothesis that fluoride levels in plaque and enamel, achievable through use of fluoridated water and/or fluoride dentifrices, may produce appreciable inhibition of glycolysis at the acidic pH levels which are readily achieved in plaque. Thus, bacterial acid production may activate plaque and enamel-bound fluoride, resulting in inhibition of further acid production, and thereby contribute substantially to the other cariostatic mechanisms of fluoride.*

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Introduction.

The cariostatic effect of fluoride is primarily attributed to a variety of physical and chemical mechanisms, including systemic effects on pre-eruptive tooth development (Aasenden and Peebles, 1974) and the reduction of enamel solubility at acidic pH levels (Brown *et al.*, 1977) mediated by the formation of fluorapatite (Moreno *et al.*, 1977). Topical fluorides facilitate remineralization of the enamel surface (ten Cate and Arends, 1977) and may interfere with bacterial adherence and plaque formation on enamel (Killian *et al.*, 1979). There is also substantial evidence indicating an antimicrobial and/or antimetabolic action of fluorides (Hamilton, 1977; Jenkins and Edgar, 1977; Loesche, 1982). While there is some question as to whether fluoride concentrations available *in vivo* are sufficient to produce a bactericidal effect in plaque, there is a distinct possibility that enough fluoride is present to have an antimetabolic effect at low pH levels. There is evidence that fluoride derived from topical fluorides (Geddes and McNee, 1982) or water fluoridation (Jenkins *et al.*, 1969; Agus *et al.*, 1980) may decrease the acidogenic potential of dental plaque.

Estimates of total fluoride in plaque range from 5 to 150 ppm, and higher, but it is likely that only a small amount of this would be available to exert an antimetabolic effect (Charlton *et al.*, 1974; Jenkins and Edgar, 1977). It is not clear how much enamel-bound fluoride would be available, since experiments in which natural or artificially fluorosed enamel was added to salivary sediments (Lilienthal and Martin, 1956) or lactobacilli (Bibby and Van Kesteren, 1940; Briner and Francis, 1962; Zwemer, 1957) have produced contradictory results, possibly because of the use of different pH levels in these assays. The potential role of fluoride-enriched enamel in the inhibition of plaque acidogenesis is thus unclear at this time. The present study was undertaken to assess the ability of fluorapatite as well as fluoride ion concentrations approximating those found in plaque to inhibit lactic acid generation by resting cell suspensions of plaque bacteria *in vitro*.

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Materials and methods.

Resting cell suspensions of the various oral bacteria were assayed for lactic acid production from 0.1% sucrose by means of an adaptation of the method of Minah and Loesche (1976), as described previously (Harper and Loesche, 1983). Reactions were carried out anaerobically in 250-μL volumes in 2-dram vials at 35°C. Representative strains of *Streptococcus mutans* and other plaque isolates were selected from stocks maintained at the University of Michigan as blood suspensions frozen on glass beads (Nagel and Kunz, 1972). *Streptococcus mutans* strains E49 (serotype *a*), FA1 (serotype *b*), Ingbritt (serotype *c*), OMZ 176 (serotype *d*), and LM7 (serotype *e*), as well as *Streptococcus sanguis* 10558 and *Actinomyces viscosus* strain 21, were used.

Except where noted, resting cell suspensions were made in Reduced Transport Fluid (RTF) containing 0.98 mmol/L EDTA (Calbiochem) in order to inhibit cation-dependent clumping of *S. mutans* cells and to bind apatite-derived calcium. The FAP powders used in these studies were prepared by the procedure described by Crommelin *et al.* (1983), and were thoroughly washed with glass-distilled de-ionized water to remove free fluoride. The FAP powders contained approximately 2000 μg/g fluoride, while the HAP powders contained no detectable fluoride. Two and one-half mg of HAP and/or FAP powders were added to the reaction mixture just prior to the start of the assay. The importance of EDTA in the reaction mixture was shown in experiments in which the RTF was formulated without a basic buffer component and without EDTA. Although the RTF was unbuffered, final pH levels were only 0.2 to 0.4 pH units below starting values in the pH 6.5 suspensions; there was minimal pH drop in the pH 4.5 suspensions. Fluoride concentrations in the apatite-containing suspensions were determined at the end of the assay, after the suspension was filtered through a 0.22-μm filter and diluted in TISAB buffer.

Subsequent experiments evaluated the amount of NaF needed in buffered, FAP/HAP-free reaction mixtures to approximate the amount of inhibition observed in FAP-containing suspensions. At 5, 15, and 30 minutes, 1-μL aliquots of the assay suspensions were spotted on thin-layer chromatography sheets. The sheets were dried and developed, spots representing lactic acid and sugar were removed, and radioactivity was determined in a liquid scintillation counter. Previous studies by Minah and Loesche (1976) showed that resting cells of these species incubated with 0.1% sucrose at pH 7 produced significantly more lactic acid than acetic, butyric, or propionic acid. Lactic acid production was thus considered representative of fermentative activity, and other acidic end-products were not quantitated.

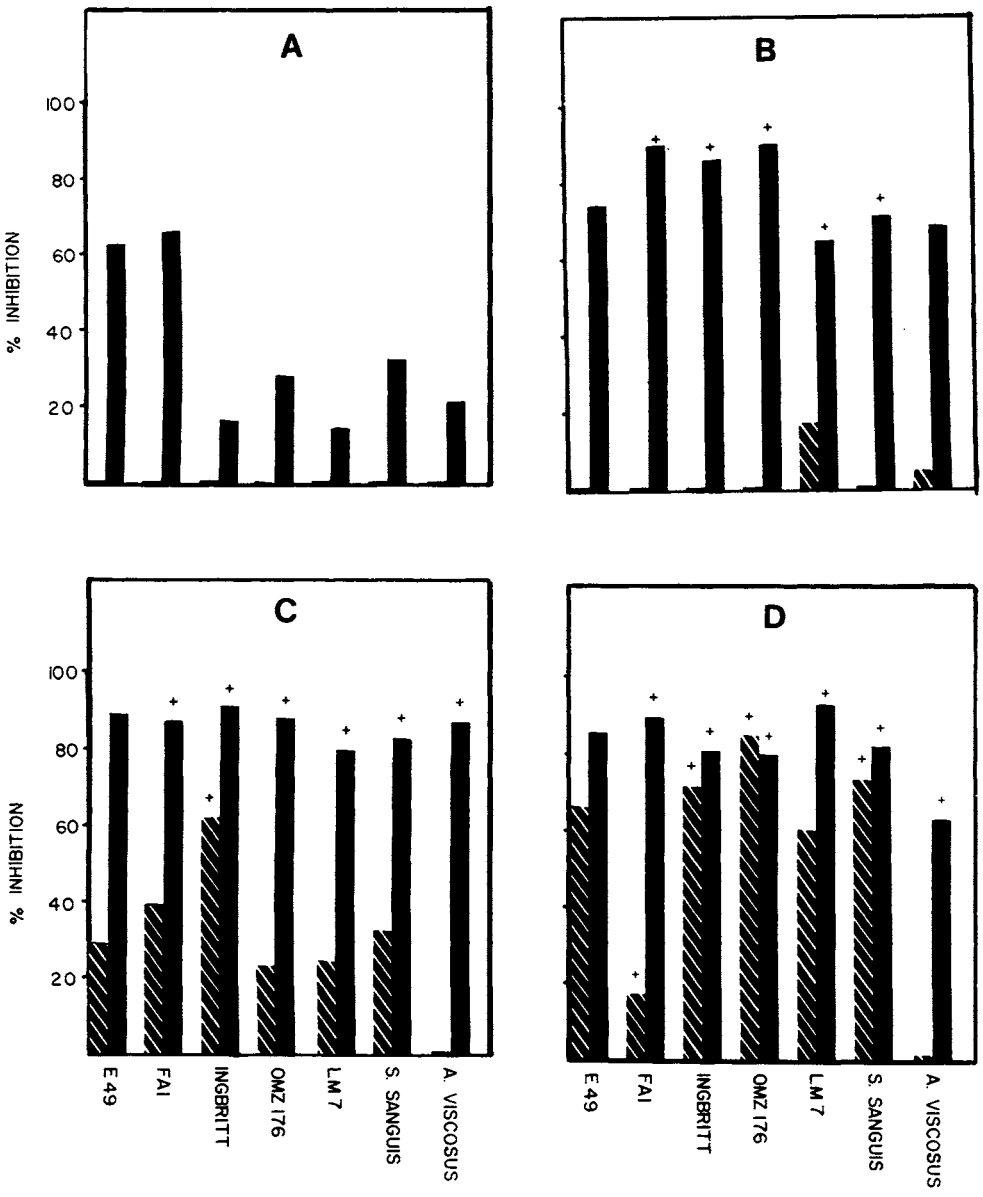

Fig. — Inhibition of lactic acid production from 0.1% sucrose in resting cell suspensions of *S. mutans* strains E49, FA1, Ingbritt, OMZ 176, and LM7, *S. sanguis* 10558, and *A. viscosus* strain 21 incubated with 0.5 (A), 2.3 (B), 11.3 (C), or 22.6 (D) μg/mL of fluoride at pH 6.5 (broken bars) or pH 5.0 (solid bars). Percent inhibition = 100 − [(acid production in fluoride-containing suspensions ÷ acid production in fluoride-free suspensions) × 100]. A + indicates statistically significant inhibition relative to fluoride-free suspensions ($P < 0.05$, Mann-Whitney U Test).

Each assay was performed from four to six times. Data were normalized to the number of total colony-forming units (CFU) present in the suspensions. Significant differences between FAP- and HAP- containing suspensions were determined by paired Student *t* tests. Statistical significance among groups incubated with NaF was tested by the Mann-Whitney U test.

Results.

Inhibition of acid production by FAP. — Suspensions incubated with FAP at pH 6.5 in the absence of EDTA produced approximately 10 to 20% less acid at 15 and 30 minutes than did suspensions incubated with HAP (Table 1). At pH 4.5, there was a statistically significant ($p < 0.05$) 18 to 25% reduction in acid production in the suspensions incubated with FAP in comparison with those incubated with HAP. Differences observed at five minutes were not statistically significant.

In this assay system, any calcium released from the apatites would remain in solution, thereby retarding further demineralization by a common ion effect. To confirm the importance of FAP demineralization to its inhibitory effect upon glycolysis, 0.98 mmol/L EDTA was added to the HAP and FAP suspensions. The inhibitory effect of FAP was sharply increased under these conditions for *S. mutans* and *S. sanguis* (Table 2). At pH 6.5, acid production by strain Ingbritt in the FAP suspensions was significantly decreased after 15 and 30 minutes of incubation, and there was a significant 85% inhibition of acid production in the presence of FAP at pH 4.5 at all time periods. Addition of FAP to resting cell suspensions of *S. mutans* E49 (Table 2) and *S. sanguis* 10558 (Table 2) also resulted in inhibition of acid production, comparable to that observed in Ingbritt cells incubated under the same conditions.

Studies were performed in which both HAP and FAP were added to the same reaction mixture to confirm that the inhibition of acid production in these resting cell suspensions was mediated by fluoride released from dissolution of the FAP. Since HAP is more acid-soluble than FAP (Brown *et al.*, 1977), it was assumed that the acids produced by the bacteria would preferentially dissolve the HAP in mixtures of HAP and FAP powders and thereby delay or minimize the release of fluoride. Resting cell suspensions of *S. mutans* Ingbritt incubated with a mixture of 1.5 mg HAP and 1.0 mg FAP did not exhibit inhibition of acid production from sucrose in comparison with cells incubated with 2.5 mg of HAP (Table 3). This indicates that demineralization of the FAP was required for the inhibition to occur, since significant inhibition could be obtained in cell suspensions incubated with as little as 0.25 mg FAP in the absence of HAP (Table 4).

The reaction mixtures incubated at pH 4.5 with 2.5 mg FAP were analyzed with a fluoride electrode and found to contain

TABLE 1
LACTIC ACID PRODUCTION BY RESTING CELL SUSPENSIONS OF *STREPTOCOCCUS MUTANS* STRAIN INGBRITT IN THE PRESENCE OF HYDROXYAPATITE (HAP) OR FLUORAPATITE (FAP)

pH		Time 5 min	15 min	30 min
		ηM acid per 6 × 10^7 CFU		
6.5	HAP	57.6 ± 5.9	110.2 ± 5.9	188.3 ± 1.1
6.5	FAP	63.0 ± 9.7	100.3 ± 5.9	173.8 ± 8.7
4.5	HAP	74.6 ± 7.2	117.3 ± 3.8	213.4 ± 2.5
4.5	FAP	61.3 ± 19.6	94.0* ± 4.1	160.9* ± 9.9

Resting cell suspensions were incubated in the presence of 2.5 mg HAP or FAP powder added at start of assay.
Suspensions were unbuffered and did not contain EDTA.
Values represent mean of four replicates ± standard error.
*FAP significantly lower than HAP ($P < 0.05$, Student's *t* test).

TABLE 2
LACTIC ACID PRODUCTION BY RESTING CELL SUSPENSIONS OF *S. MUTANS INGBRITT*, *S. MUTANS* E49, OR *S. SANGUIS* 10558 IN THE PRESENCE OF 0.98 mmol/L EDTA AND 2.5 mg HYDROXYAPATITE (HAP) OR FLUORAPATITE (FAP)

pH		Time 5 min	15 min	30 min
		ηM acid per 6 × 10^7 CFU		
		S. mutans Ingbritt		
6.5	HAP	18.2 ± 1.8	52.8 ± 4.3[a]	68.2 ± 3.6
6.5	FAP	23.4 ± 3.5	35.9* ± 2.7[a]	39.4* ± 5.1
4.5	HAP	13.1 ± 4.4	25.5 ± 6.5[a]	29.0 ± 5.2
4.5	FAP	4.2* ± 1.1	3.9* ± 0.5[a]	4.2* ± 0.3
		S. mutans E49		
6.5	HAP	23.9 ± 2.6	39.6 ± 1.1	80.0 ± 4.0
6.5	FAP	22.4 ± 2.1	36.5 ± 0.8	63.4* ± 0.9
4.5	HAP	11.7 ± 1.0	23.5 ± 1.7	38.0 ± 2.4
4.5	FAP	3.4* ± 0.4	5.0* ± 1.0	5.4* ± 1.0
		S. sanguis 10558		
6.5	HAP	95.1 ± 8.0	203 ± 19.6	300 ± 31.2
6.5	FAP	51.4* ± 8.0	99* ± 12.7	150* ± 11.4
4.5	HAP	22.4 ± 0.6	38.1 ± 1.3	62.4 ± 2.2
4.5	FAP	11.9* ± 3.3	17.4* ± 6.2	10.9* ± 2.8

Values represent mean of from four to eight replicates ± standard error.
All suspensions contained 0.98 mmol/L EDTA.
[a]These data taken at 20, not 15, minutes.
*FAP significantly lower than HAP ($P < 0.05$, Student's *t* test).

approximately 5 to 10 μg/mL of free fluoride ion. In order to verify that this level of fluoride could be inhibitory, resting cells of *S. mutans* and several other acidogenic plaque species were incubated with sodium fluoride sufficient to give from 0 to 22.5 μg/mL of fluoride. Addition of 0.5 μg/mL of fluoride (Fig., A) did not inhibit acid production at pH 6.5, but produced a 20 to 60% inhibition of acid production at pH 5.0 in *S. mutans* strains E49, FA1, and OMZ 176, *S. sanguis* 10558, and *A. viscosus* strain 21.

Addition of 2.3 μg/mL of fluoride (Fig., B) to suspensions incubated at pH 6.5 produced little or no inhibition of acid production, while this amount of fluoride caused a 60 to 80% inhibition of acid production at pH 5.0. This inhibition was statistically significant ($P < 0.05$, Mann-Whitney U test) for *S. mutans* strains FA1, Ingbritt, OMZ 176, and LM7, as well as for the *S. sanguis* strain. Addition of 11.3 μg/mL of fluoride to the pH 6.5 suspensions (Fig., C) caused a 20-60% inhibition

TABLE 3
LACTIC ACID PRODUCTION BY RESTING CELL SUSPENSIONS OF *STREPTOCOCCUS MUTANS* STRAIN INGBRITT IN THE PRESENCE OF HYDROXYAPATITE (HAP) AND FLUORAPATITE (FAP), ALONE AND IN COMBINATION

pH		Time 5 min	15 min	30 min
		ηM acid per 6 × 10^7 CFU		
6.5	2.5 mg HAP	78.0 ± 7.9	111.4 ± 12.7	191.6 ± 34.5
6.5	1.5 mg HAP + 1.0 mg FAP	72.1 ± 9.4	130.7 ± 21.2	202.2 ± 27.8
4.5	2.5 mg HAP	40.3 ± 2.5	75.6 ± 6.7	137.5 ± 11.9
4.5	1.5 mg HAP + 1.0 mg FAP	53.4 ± 3.1	72.3 ± 7.9	131.8 ± 10.2

Values represent mean of four replicates ± standard error.
All suspensions contained 0.98 mmol/L EDTA.

TABLE 4
LACTIC ACID PRODUCTION BY RESTING CELL SUSPENSIONS OF *STREPTOCOCCUS MUTANS* STRAIN INGBRITT IN THE PRESENCE OF HYDROXYAPATITE (HAP) OR FLUORAPATITE (FAP)

pH		Time 5 min	15 min	30 min
		ηM acid per 6 × 10^7 CFU		
6.5	0.25 mg HAP	16.0 ± 0.9	30.3 ± 2.2	44.7 ± 3.3
6.5	0.25 mg FAP	18.6 ± 1.6	28.1 ± 1.8	39.5 ± 4.8
4.5	0.25 mg HAP	32.5 ± 8.2	52.0 ± 15.6	82.0 ± 24.6
4.5	0.25 mg FAP	14.9* ± 0.9	25.0* ± 1.4	36.6* ± 3.8

Values represent mean of four replicates ± standard error.
* FAP significantly lower than HAP ($P < 0.05$, Student's *t* test).
All suspensions contained 0.98 mmol/L EDTA.

of acid production by the six streptococcal strains, but did not affect acid production by *A. viscosus*. However, all strains were significantly inhibited by 11.3 μg/mL F^- at pH 5.0. Acid production by all streptococcal species, but not *A. viscosus*, was inhibited by 22.6 μg/mL of fluoride at pH 6.5 (Fig., D). At pH 5.0, 22.6 μg/mL of fluoride significantly inhibited lactic acid production in all strains, including *A. viscosus*.

Discussion.

The antimetabolic effect of fluoride in salivary sediments and pure cultures of oral bacteria has been well documented (Hamilton, 1977), as has the enhancement of fluoride-mediated inhibition at low pH levels (Jenkins, 1959; Beighton and Hayday, 1980; Hamilton, 1977; Schuster *et al.*, 1981). Geddes *et al.* (1982) have indicated that daily rinsing with 0.2% NaF may decrease the acidogenic capacity of plaque. Jenkins *et al.* (1969) and Agus *et al.* (1980) have indicated that plaque from individuals consuming fluoridated water is less acidogenic *in vitro* than plaque from individuals living in non-fluoridated areas. The present results support these studies, showing that, at pH 5.0, fluoride levels as low as 0.5 μg/mL could cause a 60% inhibition of acidogenesis by selected strains of *S. mutans*, and concentrations of 2.3 μg/mL or higher significantly reduced lactic acid production by all tested *S. mutans* strains. These findings support the hypothesis that the levels of free fluoride found in plaque may have an antimetabolic activity *in vivo* during times when plaque is acidic.

The observation that FAP could inhibit acid production by *S. mutans* and other plaque bacteria at low pH levels confirms

the suggestions of previous investigators that enamel-bound fluoride may be inhibitory *in vivo* under demineralizing (*i.e.*, low pH) conditions. The increased inhibition of acid production by the inclusion of EDTA, coupled with the decrease in inhibition when HAP was mixed with FAP, indicated that demineralization of FAP was necessary for expression of FAP-mediated inhibition. Thus, any procedure which incorporates fluoride into dental enamel may have a cariostatic effect not only by reducing the enamel solubility, but also by acting as a depository for potentially antimetabolic reserves of fluoride which at low pH levels can be released in concentrations adequate for inhibition of acid production.

The approximately 2000 μg/g concentration of fluoride in the FAP used here was similar to the amount of fluoride present in fluorotic teeth (DeAisenberg and Ubios, 1974) or teeth recently treated with acidulated phosphate fluoride (DeAisenberg and Ubios, 1974), but is higher than the 25 to 1000 ppm range found in teeth from water-fluoridated areas (DeAisenberg and Ubios, 1974; Weatherell, 1977). It should be noted that, since the fluids used in these studies had minimal levels of calcium and phosphate, demineralization of the apatite and release of F^- from FAP may have been significantly higher than might be expected *in vivo*. Thus, enamel with lower proportions of FAP may not by itself contain enough readily available fluoride to provide a consistent antimetabolic effect upon the plaque flora. However, plaque-associated fluoride, which is increased by water fluoridation (Dawes *et al.*, 1965; Charlton *et al.*, 1974; Jenkins and Edgar, 1977) and the use of fluoride dentifrice (Birkeland, 1980), may be an additional and possibly more available source of potentially antimetabolic fluoride. This hypothesis is supported by several studies (Agus *et al.*, 1980; Birkeland and Charlton, 1976) which have shown that fluoride ions are released from plaque following acidification, suggesting that the bioavailability as well as the antimetabolic potential of plaque-associated fluoride may be increased by low pH levels. The combination of very small amounts of fluoride ion derived from tooth enamel and plaque as a result of microbial acid production could serve as a metabolic damper against further sustained production of acid to the extent associated with enamel demineralization.

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